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SYNTHETIC DIETS

Their Use as a Diagnostic Procedure in Allergic Disease

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INTRODUCTION

THE evaluation of the role of ingested foods in the production of allergic symptoms is difficult and present methods of approach are frequently unsatisfactory. It is well established that the usual cutaneous and intracutaneous tests with food allergens are of little practical value in this regard, for it is generally agreed that positive skin tests do not necessarily indicate clinical sensitivity.[6] It is equally apparent that opinion regarding the incidence and importance of foods as allergens varies widely.[5, 14, 15]

One of the basic principles used both in the diagnosis and treatment of allergic disease is that removal of the patient from the offending allergen is followed by remission of the symptoms which resulted from the exposure to that allergen. Therefore if the symptoms of which any patient complains are due primarily to ingested allergens and if said patient can be maintained on an intake free of offending agents, the symptoms should disappear. This thesis is the basis for the use of most rotational and elimination diets. Furthermore, if patients could be maintained upon a nutrient preparation to which they could not become sensitized, the question as to whether or not food factors are important etiologically in the production of symptoms in any given case could be answered finally and decisively in a short time. In an effort to take advantage of these principles, various nutrient preparations of low allergenic potential have been used previously. Hill[3] used Amigen in combination with Dextrimaltose, olive oil, arrowroot starch, brewers' yeast, and various minerals as a substitute food for milk-sensitive infants with eczema. The proteins and amino acids were assumed to be nonallergenic, and the author noted that the preparation was negative upon skin test in patients with a positive skin reaction for casein. In thirty-six patients to whom this preparation was given, nineteen had a satisfactory result, that is, the food was well taken, the eczema improved, and there was weight gain. In nine patients the procedure was unsatisfactory, and in the remaining cases results were inconclusive. Hampton[4] used a similar preparation for two patients with purpura and obtained relief of symptoms in both. Olmsted, Harford, and Hampton[18] reported the use of an enzymatic casein digest in combination with a fat (corn, olive, or cottonseed oil), dextrose, minerals, and gelatin for nine patients with various allergic disorders. After a seven day

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trial of the diet, they included or excluded foods as etiological factors in the production of symptoms. Glynn[26] mentioned the use of protein hydrolysates in infants with severe eczema, and stated that 50 per cent of his patients improved.

With such a background in mind, numerous patients have been maintained on synthetic diets at the University Hospital in an effort to evaluate more definitely the role of ingested allergens in the production of allergic symptoms.

METHOD OF STUDY

Several formulas were used in the study of the cases which will be enumerated below. The first preparation used was Amigen, the pancreatic hydrolysate of casein, 77 per cent of which was in the form of simple amino acids and 23 per cent as short chain polypeptides. Pork or sheep pancreas was used for the enzymatic digestion. All of the known essential amino acids were present in the product, 150 Gm. of which contained two-thirds of the daily mineral requirement except for magnesium and potassium. A great volume of work has been done on the oral and parenteral administration of Amigen and other protein hydrolysates. It appears to have been established that Amigen will not sensitize laboratory animals to subsequent injections of skimmed milk, pancreatic extract, or Amigen itself,[9, 18, 27] that protein hydrolysates are absorbed from the gastrointestinal tract following oral administration,[28] and that excess amounts of nitrogen are not recovered from either urine or stool following oral or parenteral administration.[2, 7, 11]

Amigen was prepared for serving by dissolving 100 Gm. of the powder in 1 liter of hot water to which 225 Gm. of cane sugar were added. This was served over cracked ice in four equal portions at regular intervals throughout the day. The mixture contained 1,200 calories and the equivalent of 75 Gm. of protein. A greater quantity was occasionally used if it was thought advisable to keep the intake at higher caloric level. Some patients preferred Amigen and water without added sugar. In these instances the specified amount of carbohydrate was eaten separately as desired during the course of the day. More recently, in some cases Protolysate* has been used in lieu of Amigen. The only essential differences between the two was that the enzymatic activity utilized in the production of Protolysate was derived from fish caeca.[29]

The second formula used contained Amigen in combination with Dextrimaltose and olive oil.† A third preparation consisted of Amigen, dextrose, and certain synthetic vitamins.‡ The general composition and caloric equivalents of each of these preparations have been compiled in Table I.

TABLE I

FORMULA	CONTENTS		PROTEIN (GM.)	CARBOHYDRATE (GM.)	FATS (GM.)	CALORIES PER LITER
1	Amigen (or Protolysate)	100Gm.	75	225	0	1,200
	Cane sugar	225Gm.				
	Water to make	1,000c.c.				
2	Mead-Johnson No. 232	454Gm.	67.5	250	85	1,942
	Water to make	1,000c.c.				
3	Mead-Johnson No. 211	454Gm.	64	354	0	1,672
	Water to make	1,000c.c.				

*Mead Johnson & Company.

†Mead-Johnson Laboratory Product #232: Dextrimaltose 43.95 per cent, olive oil 18.70 per cent, Amigen 20 per cent, starch 10.39 per cent, calcium gluconate 3.64 per cent, other mineral salts and vitamins 3.32 per cent (monobasic potassium phosphate, dibasic potassium phosphate, calcium hydroxide, potassium chloride, magnesium oxide, thiamin, riboflavin, niacinamide).[29]

‡Mead-Johnson Laboratory Product #211: dextrose 77.86 per cent, Amigen 18.99 per cent, other mineral salts and vitamins 3.15 per cent (sodium chloride, calcium carbonate, dibasic calcium phosphate, dibasic potassium phosphate, ferrous sulfate, thiamin, riboflavin, ascorbic acid, citric acid).[29]

All patients had a complete study which included history, general physical examination, and routine laboratory work (serology, chest x-ray, urinalysis, complete blood count). An allergic survey followed which included an allergic history, complete cutaneous and intracutaneous skin tests in most cases, and other studies (vital capacity, cytology of exudates) if indicated. Before starting a patient on a trial of synthetic diet, the possibility of allergic symptoms arising from causes other than ingestants was excluded insofar as possible by the following practices.

(1) A period of adjustment in the hospital (four to six days) was completed to permit clearing of any symptoms which might have been the result of environmental factors.

(2) An anti-dust plan was instituted in forty of the fifty-one cases. Among the other patients there was neither clinical story nor positive skin test to suggest dust sensitivity, and symptoms were those most unusually due to dust allergy.

(3) Acute or chronic, active respiratory infection, if present, was treated by chemotherapy and/or antibiotics until maximal response was obtained.

(4) Pollen sensitive patients were not evaluated on synthetic diets during the specific pollen season.

Finally, two other precautions were observed in the effort to minimize interference with the trial of diet.

(1) Symptomatic management was simplified as much as possible so that the result of diet could be more readily evaluated. For instance, effort was made to control asthma with but one symptomatic drug, so that the number of doses required daily served as a convenient measure of response.

(2) Collateral therapy was eliminated during this period if possible. This was frequently difficult, particularly in the case of diffuse cutaneous disease.

If the program of synthetic diet was initiated, patients were managed thereafter as follows. Diet was continued for a period of ten days as a general rule. If there was definite remission of symptoms before the ten days had elapsed, sometimes the course was shortened; if there was equivocal response at the end of ten days, the diet occasionally was continued for a longer time. If there was no symptomatic response whatsoever in the ten days' time, dietary management of the patient was abandoned and food allergy was considered a most unlikely etiology for the patient's complaints.

When patients showed a definite remission of symptoms upon the synthetic diet, one of two courses was followed. Single food additions could be made at three- or four-day intervals if the purpose was to identify single allergens. In such cases, foods held in clinical suspect were added initially. If, however, the patient's clinical condition necessitated more rapid return to an adequate natural diet, food additions were multiple, in groups of two to four, and foods thought to be innocuous were added first. Factors which conditioned this decision were the clinical condition of the patient, the severity of prior symptomatology, and the advisability of reprecipitating it.

CASE SELECTION

All patients selected for trial of diet in this study were experiencing daily symptoms of an incapacitating nature. They all had been surveyed as previously described, and if symptoms persisted which were thought possibly to be allergic in origin, synthetic formulas were advised to aid in the evaluation of the role of ingestants in the production of their symptoms. Thus in many instances the program was used because inhalant and drug allergens had been reasonably excluded and symptoms had persisted. Despite case selection by a method which had few positive criteria, the number of patients for whom the program was advised did not increase rapidly. In 1946-1947, over an eighteen-month period, there were but fifty-one such instances. Final diagnoses in these cases have been tabulated in Table II.

TABLE II

Perennial rhinitis	1
Bronchial asthma	26
Bronchiectasis*	1
Emphysema*	2
Chronic urticaria	2
Atopic eczema	1
Chronic ulcerative colitis	3
Cephalalgia	2
Allergic colitis	1
Vascular allergy	1
Chronic conjunctivitis	1
Erythema multiforme	1
Ulcerative stomatitis	1
Periarteritis nodosa	1
Disseminated lupus†	1
Total	51

*Admitting diagnosis was bronchial asthma.

†Discharge diagnosis uncertain; disseminated lupus considered the most likely. Biopsy not conclusive.

Multiple diagnoses were the rule in most cases, reflecting the well-known tendency for allergic disease to affect more than one organ system, but the predominating symptom-complex was used for the purpose of the classification in Table II. It should be emphasized that of the fifty-one patients put on the synthetic diets, forty-eight were hospitalized.

OBSERVATIONS

No diagnostic or therapeutic measure is of general value, however accurate or beneficial it may be, unless it can be applied readily to most patients for whom it may be indicated. It must, therefore, be admitted that these formulas were almost uniformly distasteful to patients, and those on synthetic diet programs required complete explanation initially and continued moral support throughout the trial period. In our experience, no one of these formulas was definitely more acceptable than any one of the others. However, against the initial objection on the part of most patients stands the fact that of the fifty-one to whom the diet was offered, all but three attempted the trial. Of the three patients refusing to continue the program after their initial meal, one had a vague cephalalgia later diagnosed as a functional problem by the psychiatric consultant, one had an eczema eventually diagnosed as neurodermatitis by the dermatologist, and one had bronchial asthma of moderate severity.

Four other patients developed nausea, vomiting, and/or diarrhea during the course of the trial period which necessitated interruption of the program. All four of these patients developed their symptoms on, or slightly before, the seventh day. In the records of nine other patients there was note of slight nausea and vomiting, the occasional refusal of a feeding, or mild diarrhea. The program was completed in all the latter instances.

A second general aspect of the situation to which attention had to be directed was the over-all effect of these formulas upon the patients. Elimination and rotational dieting have been rightfully criticized by some because their

use frequently leads to inadequate intakes and depleted nutritional states. Since the trial period was a short one, after completion of which effort was made to restore an adequate, balanced intake to the patient, it was not felt that the trial would precipitate deficiency states. Caloric intake was kept at 1,200 calories or above. In thirty-eight instances where there was adequate and accurate record of body weight, twenty-six patients lost an average of 4.2 pounds each, five patients gained an average of 2.1 pounds each, and in seven instances there was no change in body weight. In no instance was there any clinically demonstrable detrimental effect upon the patients other than the gastrointestinal symptoms noted. These symptoms quickly subsided upon termination of the diet.

Considerable difficulty was experienced in evaluating the specific result of trial of diet upon allergic manifestations. Despite the fact that the plan appeared to lend itself to precision in this respect and to the development of objective criteria for making such decisions, changes were frequently relative and subjective responses had to be taken into consideration. Responses to diet trial, however, could be grouped into the following four categories.

(1) Patients in whom there appeared to be no food factor. These patients completed ten days of diet without change in their symptoms.

(2) Instances in which there appeared to be a definite food factor. Symptoms of these patients cleared while on synthetic diet and were reproduced by additions to the formula.

(a) If additions were single and flare resulted, single allergens were identified.

(b) If groups of foods were added with resultant flare, a definite food factor was considered present, even though a specific allergen was not identified.

(3) A small group of patients who experienced complete remission of symptoms upon the diet, but who subsequently tolerated return to general diet without reappearance of symptoms.

(4) Instances in which trial of diet was unsatisfactory for one of the following reasons:

(a) Failure to complete the course.
(b) Uncontrolled collateral therapy.
(c) Equivocal result.
(d) Inadequate follow-up contact with patient.

Table III summarizes the results in fifty-one patients who received synthetic diets.

In Group 1 foods were presumptively eliminated as a cause of symptoms. The fifteen patients in Group 2 had a definite food factor. It follows, therefore, that in thirty-eight instances information of benefit to both patient and physician was obtained.

In the group of fifteen patients in whom there was thought to be a definite food factor, twenty-three separate allergens were diagnosed. Milk was the most frequent offender (eleven times); wheat, white potato, and

TABLE III

DIAGNOSIS	GROUP 1	GROUP 2	GROUP 3	GROUP 4	TOTAL
Perennial rhinitis	1				1
Bronchial asthma	12	5	1	8	26
Pulmonary emphysema	2				2
Bronchiectasis	1				1
Atopic eczema		5		2	7
Chronic urticaria	2				2
Chronic ulcerative colitis	1	1	1		3
Allergic colitis		1			1
Cephalalgia	1			1	2
Erythema multiforme	1				1
Vascular allergy		1			1
Chronic conjunctivitis	1				1
Chronic ulcerative stomatitis	1				1
Lupus erythematosis		1			1
Periarteritis nodosa		1			1
Total	23	15	2	11	51

beef were next (each three times). Not all patients in this group had been skin tested, but the majority had. For fifteen of the twenty-three allergens demonstrated, there were both scratch and intracutaneous tests for comparison. In but four of the fifteen instances were there positive tests, either scratch (one) or intracutaneous (three), of *any* degree of positivity. This lack of correlation between skin testing and clinical observation in food allergy is in accord with general opinion as to the diagnostic specificity of skin testing for food allergens.[6,14]

DISCUSSION

Several considerations relative to the foregoing should be discussed in somewhat greater detail. First, the nonallergenicity of the formulas used cannot be assumed. The possibility remains that milk-, fish-, pork-, mutton-, or corn-sensitive patients might continue to have allergic symptoms secondary to the ingestion of minute amounts of these substances remaining unaltered and undetectable in the final product. In view of the presence of short chain polypeptides, there remains the potential danger of immunologic reaction.[26] An enzymatic casein digest will not sensitize laboratory animals,[9, 18, 27] and clinical observations supporting the theoretic nonallergenicity of these preparations have been made. For instance symptoms of patients known to be milk-sensitive have been observed to clear upon an enzymatic digest of casein, and later to flare with the addition of milk to the basic formula. The final answer to this question should be withheld pending further clinical investigation and trial.

It should be re-emphasized that unpalatability is the chief objection encountered in the use of these diets. Our results have not been tabulated for each of the formulas because practical differences between them with respect to acceptability or clinical result were not apparent. It seems doubtful that any patient with less than major allergic symptoms would elect to remain upon such a program for the full trial period. Because of this impression, the procedure almost uniformly was reserved for inpatients.

The importance of controlling environmental factors, the dust allergen, and concomitant infection prior to embarking upon a diagnostic dietary regime cannot be overemphasized. On several occasions it was observed that proper consideration of these factors obviated the necessity for dietary trial which had originally been contemplated. The presence of an active infectious process, at least in the respiratory tree, contraindicates a trial of one of the synthetic formulas. For example, one patient with bronchiectasis superimposed on an allergic asthma could not be controlled from the standpoint of the asthma by any means during a flare of the pulmonary infection. After control of the pneumonitis with antibiotics and postural drainage, residual asthma cleared rapidly upon institution of a synthetic diet.

It again should be pointed out that patients for whom diet trial was ordered were those whose symptoms were more than moderately incapacitating. The total number forms but a small, though unestimated, percentage of the cases seen in Allergy Clinic.

The cause of gastrointestinal symptoms secondary to the use of these formulas is somewhat obscure. Aspartic and glutamic acids are reported to cause nausea and vomiting in both man and laboratory animals,[22, 23] and casein hydrolysates contain both of these amino acids.[25] This may explain the development of symptoms, but delay in their appearance until the sixth or seventh day of dieting is less readily explained on this basis than if appearance was more immediate. One patient, who developed nausea and vomiting with Amigen orally, was maintained satisfactorily on intravenous Amigen and subsequently tolerated a second trial of the oral preparation for a ten-day period without the development of gastrointestinal tract symptoms. Although this patient was sustained with the parenteral preparation when unable to tolerate Amigen orally, it was not the general policy to follow this plan. The end did not appear to justify the means even in the face of considerable literature attesting to the safety and efficacy of parenteral amino acid administration.[1, 8, 10, 12, 13, 17, 19, 20, 21, 24]

Finally, it should be kept in mind that the trial of diet occasionally was used as a differential diagnostic procedure in syndromes of obscure etiology. The final diagnosis was in some cases a condition not commonly thought to be of allergic origin. These cases have been included irrespective of this consideration because they represented instances in which synthetic diets were utilized in differential diagnosis. The recorded results (Table III) usually reflected the accepted nonallergic etiology of the disorder (namely pulmonary emphysema, bronchiectasis), but in two instances (lupus erythematosis, periarteritis nodosa) there was apparent benefit from the formulas in disease entities not commonly held to be allergic in origin. The patient with lupus erythematosis was a middle-aged, white man who was the victim of a disseminated process affecting most of the major organ system. A skin biopsy was reported as compatible with lupus, but a skin-muscle biopsy from a different site revealed essentially normal tissues. The clinical impression at the time of discharge was probable lupus erythematosis, and follow-up contact with the patient was insufficient to verify or disprove the diagnosis. The

symptoms of this individual twice cleared upon synthetic diets and twice flared with additions. Periarteritis nodosa occurred in a middle-aged, white woman. The diagnosis was made at the time of necropsy. Prior to death the patient had had almost intractable asthma for many months. There was associated high-grade (20 to 50 per cent) eosinophilia observed in peripheral blood as well as transient pulmonary consolidations which appeared serially in different locations in the lung parenchyma. These were clearly demonstrated by successive chest x-rays. In the last six months of life, the only time of relative freedom from asthma were those periods when the patient subsisted upon synthetic formulas only. The example of this latter patient may be suggestive evidence of an allergic etiology for periarteritis nodosa, supporting Rich's recent observations.[39] The instance of probable lupus erythematosis can be regarded as nothing more than a recital of observations which at this time cannot be extended to any conclusions.

In comparison with previously reported use of synthetic diets the formulas discussed herein would appear to be potentially less allergenic than any of the others. A more varied and larger series of cases has been reviewed than previously.

CONCLUSIONS

A group of fifty-one patients suffering from various ailments thought to be entirely, or in part, of allergic origin has been studied by means of synthetic diets. In thirty-eight of these patients, or 74 per cent, it was felt that the trial afforded information of definite positive benefit to both the patient and physician, information which could not have been acquired by other methods in so short a time with equal clarity.

The method would seem to supply another worth-while, safe, and sound approach to the problem of exact diagnosis of food allergy, although at the moment, in its present form, it does not appear practicable for outpatient use or for use upon patients with less severe allergic manifestations.

Until more clinical experience and study have justified the assumption that these formulas are nonallergenic, positive diagnoses of food allergy may be more valid than the negation of its presence after trial of synthetic diet.

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THE LIFE SPAN OF THE MEGALOCYTE AND THE HEMOLYTIC SYNDROME OF PERNICIOUS ANEMIA

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THERE is now general agreement that pernicious anemia is a deficiency disease. The ability of the liver extract principle to change the megaloblastic maturation arrest to a normoblastic type of red cell production seems to demonstrate that the site of the primary action of the antipernicious factor is located in the marrow.

Patients with Addisonian anemia also show regularly a severe disturbance of the pigment metabolism. There is usually mild retention jaundice. The hemolytic index,[1] that is, the urobilinogen excretion in the feces correlated with the total mass of circulating hemoglobin, is far in excess of normal values.[2, 3]

These abnormalities of the pigment metabolism apparently are indicative of an existing hemolytic process since they also are observed in all other known hemolytic syndromes. However, contrary to these other types of hemolytic disorders, untreated cases of pernicious anemia show no increase or only a very slight increase of the reticulocytes in the circulating blood.[3] If reticulocytosis is evaluated as a measurement of red cell replacement and the high pigment output as an indication of the simultaneously existing increased erythrocyte disintegration, a considerable imbalance in favor of erythrocyte destruction would be present in untreated pernicious anemia. Consequently the peripheral blood should become rapidly depleted of erythrocytes, resulting in an early death of the patient. Clinical observations are not in agreement with such a mechanism. Therefore Addisonian anemia often has been considered not to be a true hemolytic syndrome. In order to account for the excessive bile pigment output, various explanations have been suggested. It has been postulated either that hemoglobin is destroyed in the marrow without ever entering the circulation,[4] or that the excessive urobilinogen is not derived from hemoglobin at all but represents rather pigment formed from other substances.[5] All these hypotheses tacitly imply that the morphologically abnormal megalocytes, after having reached the circulation, have then a normal survival time.[3]

In previous publications[6, 7] it has been pointed out that the common denominator of all hemolytic syndromes regardless of the great variety of causative mechanisms is to be found in a shortened life span of the various types of cells involved. In order to determine whether Addisonian anemia may be classified as a true hemolytic syndrome, estimations of the survival time of erythrocytes from patients with untreated pernicious anemia were performed in this study.

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MATERIAL AND METHODS

The diagnosis of pernicious anemia in patients used in this investigation was based on a compatible clinical picture with histamine refractory achlorhydria, the presence of a macrocytic hyperchromic anemia, and a marrow examination showing the megaloblastic type of erythrocyte maturation accompanied by giant metamyelocytes. A moderate hyperbilirubinemia was found in the untreated patients. Furthermore, all patients responded well to liver treatment.

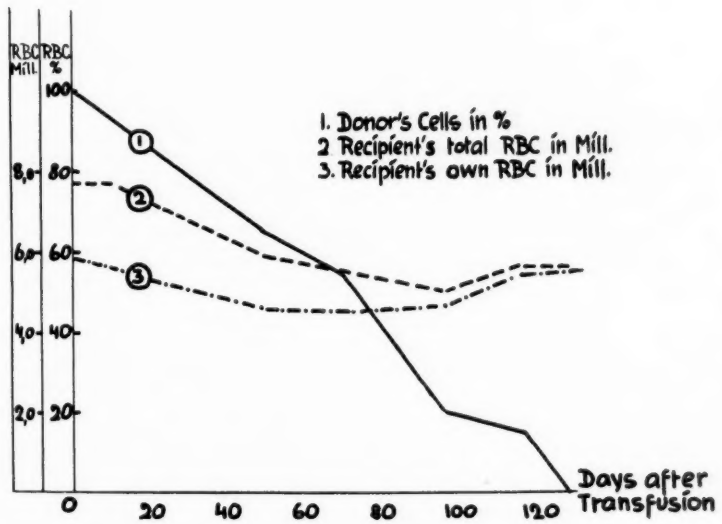

Fig. 1.—Survival time of normal red cells in artificial erythrocytosis expressed in per cent of surviving cells.

The methods used in this study have been described in detail in a previous publication.[7] Determinations of the survival time were performed with the method of differential agglutination (Ashby technique). Only young, normal children (2 to 4 years old) were selected as recipients. Because of their small circulating blood volume, a correspondingly small transfusion was found sufficient to result in a satisfactorily high addition of the donor's cells per cubic millimeter. Packed cells derived from 250 to 500 c.c. of whole blood were introduced, yielding an initial increase of 340,000 to 820,000 red blood cells in the recipient's total erythrocyte level. These initial values were established twenty-four to forty-eight hours after transfusion in order to avoid any significant inaccuracy due to an augmentation of the circulating fluid volume, although it is realized that some of the transfused cells already may have been eliminated within these first twenty-four to forty-eight hours. After determining the starting point, follow-up studies were done twice weekly during the first month and from then on once every week.

Some of the recipients showed values of 5.5 to 5.8 million red cells following transfusion. In order to ascertain whether such an abnormally high count may have an influence on the survival time of the tagged cells, normal erythrocytes were transfused into children to such an extent that the total red count was brought up to more than seven million per cubic millimeter. Such an artificial erythrocytosis has no influence on the survival time. Fig. 1 shows an experiment in which the red count had been increased from an initial level of 5.8 to 7.7 million per cubic millimeter. After all transfused cells had disappeared, the patient's red count was 5.5 million per cubic millimeter. The elimination curve of the foreign cells was a normal one and their average life span was 127 days, an entirely normal value.

RESULTS

Nowadays the scarcity of untreated cases of pernicious anemia presents a major obstacle to investigations on the pathogenic mechanisms operating in this disorder. Our study was performed on four cases only.* The hematologic data of the donors and also of the healthy recipients are compiled in Table I.

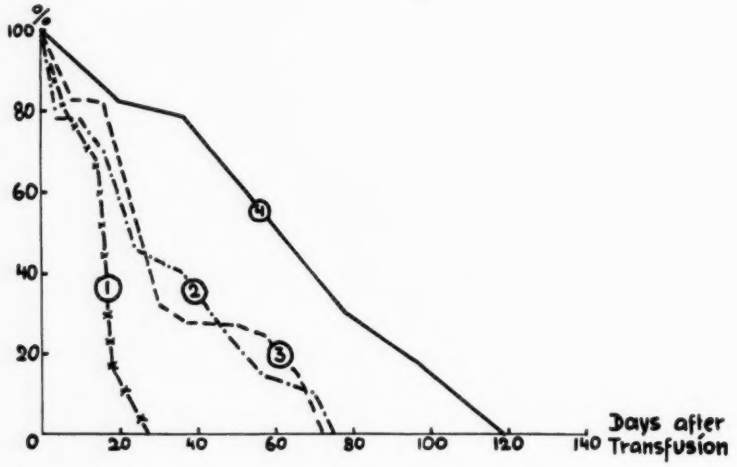

Fig. 2.—Survival time of pernicious anemia cells in normal environment expressed in per cent of surviving cells.

Fig. 2 shows the curves illustrating the disappearance of the donor's cells. The percentages of the surviving cells are plotted against the number of days following transfusion. Patients 1 and 2 were patients with pernicious anemia in relapse who had received no specific treatment for several months prior to the experiment. Their red cells were completely eliminated within twenty-eight and seventy-five days respectively when transfused into a normal environment. The profiles of their elimination curves are definitely abnormal and are similar to those seen in the other hemolytic syndromes caused by an intracorpuscular abnormality of the red cells.[7]

Patient 3 had been under continuous treatment with liver for thirteen years. Then treatment was stopped entirely for about three years. A severe degree

TABLE I. TRANSFUSION OF PERNICIOUS ANEMIA CELLS

	DONORS							
PATIENT	AGE (YR.)	SEX	HB. GM.	HB. %	R.B.C. (MILL.)	RETCS. (%)	TYPING FORMULA	AMOUNT OF BLOOD USED FOR TRANSFUSION (C.C.)
1	50	F	8.4	54	2.40	1.1	O Rh+	250
2	56	F	8.7	56	2.15	1.3	O Rh+	500
3	54	M	8.0	51	1.85	0.5	O Rh+	250
4*	69	F	15.7	101	5.60	0.8	O Rh+	500

*Normalized blood picture after extensive liver treatment.

*We are indebted to Dr. S. Portis, Dr. L. Rappolt, and Dr. S. O. Schwartz for permission to use their patients in this investigation.

of anemia developed and the patient was advised to enter the hospital. A few days prior to admission he took some "liver pills." On the day of admission hemoglobin was 6.8 Gm. (44 per cent), red count, 1.66 million, and reticulocyte count, 3.8 per cent. The reticulocytes increased to 9 per cent on the third hospital day and then came back to values below 1 per cent on the tenth day. There was also a slight rise of the hemoglobin and red cell count to 8.7 Gm. (56 per cent) and 2.11 million respectively. At the time blood was taken for the determination of the survival time, the hemoglobin had decreased to 8.0 Gm. (51 per cent) and the red count to 1.85 million. Since the antipernicious anemia principle changes the abnormal megaloblastic maturation back to a normal development of the red cells, one may assume that two different populations of erythrocytes existed in the blood of this patient at the time of the transfusion, namely red cells manufactured with the participation of the liver principle and also typical megalocytes produced in the absence of the erythropoietic factor. As can be seen from Curve 3 (Fig. 2) visualizing the elimination of the foreign cells, 68 per cent of the transfused erythrocytes disappeared within thirty days; the remaining 32 per cent were completely eliminated after seventy-two days. The profile of the curve showing a distinct biphasic character is also in agreement with the assumption of the presence of two different populations of erythrocytes in the blood of this insufficiently treated patient. The suboptimal doses of antipernicious principle may also account for the total shortened survival time observed in this particular case.

Patient 4 had a normalized blood picture because of continuous liver treatment for many years. The average survival time was 119 days, an entirely normal value. This is in agreement with the recent findings of Mollison[8] who also demonstrated a normal survival pattern of red cells obtained from adequately treated patients with pernicious anemia.

Our results, therefore, demonstrate that in untreated cases of pernicious anemia there is a shortened survival time and an abnormal elimination pattern, which becomes normalized after adequate administration of liver extract.

DISCUSSION

Determinations of the average life span of normal erythrocytes transfused into patients with pernicious anemia were performed by Ashby[9] and also by

...MIA CELLS INTO NORMAL RECIPIENTS

		RECIPIENTS					RESULTS	
		HB.						
	SEX	GM.	%	R.B.C. BEFORE TRANSFUSION (MILL.)	RETCS. (%)	TYPING FORMULA	INCREASE OF R.B.C. IN RECIPIENT PER C.MM. AFTER TRANSFUSION	SURVIVAL TIME (DAYS)
250	F	13.7	88	4.55	0.9	A Rh+	379,000	27
500	M	13.1	84	4.96	0.4	B Rh+	820,000	75
250	F	14.5	93	5.39	0.1	A Rh+	340,000	72
500	F	11.9	77	4.12	0.8	A Rh+	527,000	119

Wearn and co-workers[10] as far back as 1921. A normal survival time of these transfused erythrocytes was found to be present. Recently Mollison[8] has confirmed these observations by means of the most refined modern techniques. Therefore an extracorpuscular mechanism damaging all circulating erythrocytes at random and thus influencing the life span is not demonstrable in pernicious anemia.

The survival time of the megalocytes (pernicious anemia erythrocytes) was determined with the Ashby technique by Wearn and associates (in 1922) who transfused these abnormal red cells into another patient with pernicious anemia. No abnormal behavior of the transfused corpuscles was found in this single instance. Morawitz[11] transfused normal red cells into a patient with severe pernicious anemia to such an extent that the majority of all the erythrocytes in the patient's circulation belonged to the donor's group. Since the anemia improved for a longer period of time but no change occurred in the greatly increased pigment output in the feces, Morawitz concluded that the transfused normal erythrocytes survived much longer than the pathologic megalocytes.

In 1945 one of us[6] classified pernicious anemia as a true hemolytic syndrome caused by the presence of an intracorpuscular anomaly of the erythrocyte. It was emphasized that hemolytic syndromes are not always produced by an erythrolytic activity (immune bodies, hypersplenism) but that structurally defective erythrocytes, when exposed to the normal means of destruction, disintegrate much more rapidly than normal red cells. By using the method of cross determination of the survival time of the red cells,[6, 7] it is possible to distinguish in any given case whether an extra- or an intracorpuscular mechanism is involved. When normal erythrocytes, transfused into a recipient with a hemolytic syndrome, survive normally, whereas the patient's own red cells, transfused into a normal person, have a considerably shortened life span, an intracorpuscular abnormality may be suspected. Contrariwise, when normal red cells, transfused into the patient with the hemolytic syndrome, are as rapidly destroyed as the patient's own cells, the presence of an extracorpuscular mechanism may be assumed.

While this study was in progress, we came across a report of Loutit[12] to the Royal Society of Medicine stating that he had transfused erythrocytes from two patients with untreated pernicious anemia into normal recipients and had observed a survival time of thirty and sixty days respectively. This is quite in accordance with our observations reported in the present paper. Although the elimination curves of Loutit's and our cases are all definitely abnormal, there is a considerably wide range of the average life span which is probably explained by the individual variability of the available antipernicious principle for the manufacturing of the cells.

The demonstration of an abnormal life span of the megalocyte characterizes Addisonian anemia as a true hemolytic syndrome since the common denominator of all hemolytic disorders regardless of the great variety of causative mechanisms has been found to be a shortened life span of the various types of cells involved.

Although the shortened survival time undoubtedly accounts for an increased pigment production, the question arises whether it offers a truly satisfactory explanation for the excessively high output of bile pigment regularly observed in untreated cases of pernicious anemia.

The abnormalities of the pigment metabolism existing in this disorder have always been difficult to interpret. Watson[3] in his extensive review has critically discussed this problem. Whereas in the other hemolytic syndromes there is a high reticulocyte count indicating considerable red cell replacement, untreated cases of pernicious anemia show no increase or only a very slight increase of the reticulocytes in the circulating blood. Whipple[5] was the first to point out the great discrepancy between apparent blood regeneration and destruction in untreated pernicious anemia, and he expressed his disbelief that the increased pigment excretion represents increased hemoglobin destruction. He postulated that the excessive urobilinogen is mostly derived not from the hemoglobin of the circulating red cells but rather from an increase of a hypothetical "pigment complex." This pigment complex was considered to originate from hemoglobin derivatives, food products, and body proteins. Jedlicka[4] assumed that the elevated bile pigment output stems from hemoglobin only, which, however, is predominantly destroyed in the marrow. Both hypotheses have tacitly implied that the morphologically abnormal megalocytes, after having reached the circulation, have then a normal survival time.

Fig. 3 visualizes the hypothetical relationship between the interdependent factors involved in maintaining the erythrocyte level[6] (*A*) under physiologic conditions, (*B*) in the common hemolytic syndromes (for instance hemolytic jaundice, sickle cell anemia), and (*C*) in pernicious anemia according to the theories mentioned.

It should be emphasized, however, that Heilmeyer[13] and many other investigators (Lit. see Watson[8]) have rejected these hypotheses and maintained that the excessive urobilinogen output could be adequately explained on the basis of an increased hemoglobin destruction, particularly if the extreme hyperplasia of the marrow which exists in pernicious anemia is taken into account.

At first glance the demonstration of a shortened survival time of the megalocytes seems to make any special hypotheses concerning the increased pigment output superfluous. However, recent investigations[14] demonstrate that the whole problem is much more complicated. Shemin and Rittenberg[15] showed in 1946 that oral feeding of glycine labeled with N^{15} results in the incorporation of N^{15} in the heme molecule of the red blood cells. The labeled porphyrin remains in the erythrocytes until these cells disintegrate and is then transformed into stercobilinogen. By means of this technique, the same figure for the average life span of normal erythrocytes was obtained as previously determined with the method of differential agglutination. However, when labeled stercobilin was isolated from the feces and its amount correlated with the rate of erythrocyte disintegration, it was found that a significant portion of the normal pigment production is apparently derived from sources other than hemoglobin.[14] In one case of untreated pernicious anemia a considerable increase of this extra

pigment was also demonstrable. Therefore these findings seem to support Whipple's hypothesis. Quite obviously an entire reinvestigation of the pigment metabolism in all the hemolytic syndromes now becomes necessary. However, the new evidence does not in any way reflect upon our interpretation of the significance of the shortened survival time of the megalocytes.

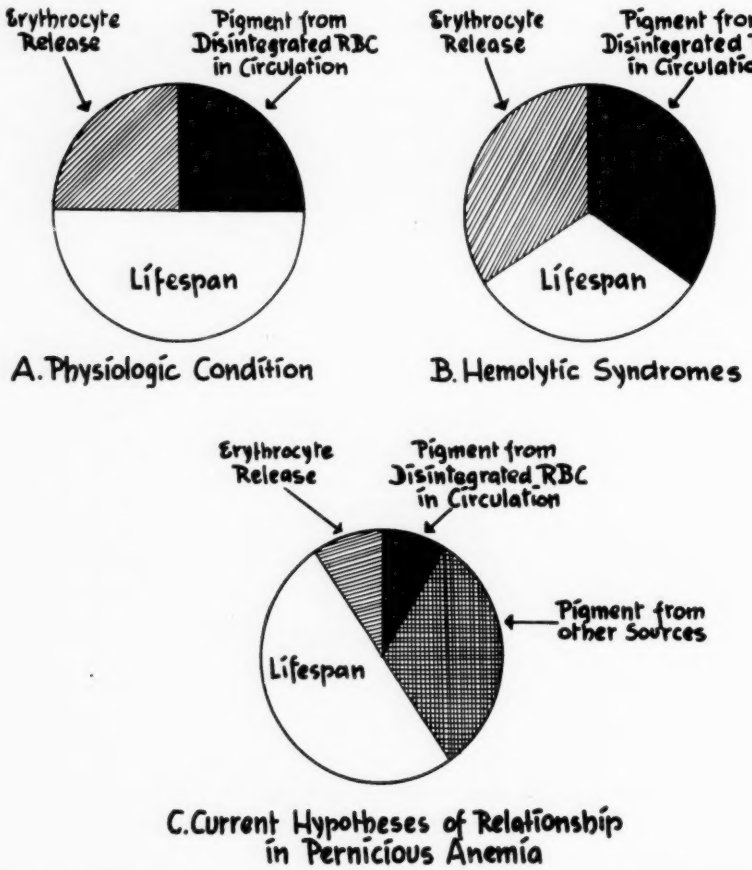

Fig. 3.—Relationship of the interdependent factors maintaining erythrocyte level.

The absence of any considerably increased reticulocyte count in untreated pernicious anemia is of particular interest. It must be assumed that in this disorder replacement of erythrocytes occurs predominantly by means of non-reticulated megalocytes.[16] Apparently completely matured although qualitatively abnormal corpuscles are released into the circulation. This is quite a distinguishing feature since no such abnormality of replacement is demonstrable in the other types of hemolytic anemia. There has been some speculation that the liver extract principle may contain a specific reticulocytogenic factor. Oliva and Frascarelli[17] report that plasma obtained from patients with a high reticulocyte count caused a definite increase in reticulocytes when injected into normal persons. This was observed with blood from patients with pernicious anemia

at the reticulocyte crises following liver treatment and also in patients with microcytic hypochromic anemia treated with iron. Oliva and Frascarelli suggest that in pernicious anemia this hypothetic reticulocytogenic factor acts in combination with the maturation principle. Somewhat against this assumption is the satisfactory hematologic response of patients with pernicious anemia treated with pteroylglutamic acid.

The demonstration that pernicious anemia is a true hemolytic syndrome does in no way invalidate the concept of this disorder as a deficiency disease. It is because of the absence of the maturation principle that defective erythrocytes enter the circulation and are then eliminated more rapidly than normal ones. Thus pernicious anemia is another example of a hemolytic syndrome caused by an intracorpuscular mechanism, namely a poorly constructed cyto-skeleton[7] of the erythrocytes.

SUMMARY

The red cells of patients with untreated pernicious anemia have a shortened survival time. After adequate treatment, the life span of the erythrocyte becomes normal.

The demonstration of a shortened survival time of the megalocyte permits the classification of pernicious anemia as a true hemolytic syndrome since the common denominator of all hemolytic anemias regardless of the great variety of causative mechanisms has been found to be a shortened life span of the various types of cells involved.

The significance of the finding of a shortened survival time for the explanation of the abnormal pigment metabolism in pernicious anemia is discussed.

Quite different from the other types of hemolytic anemia, replacement of red cells seems to occur by means of nonreticulated erythrocytes.

The demonstration that pernicious anemia is a true hemolytic syndrome does in no way invalidate the concept that this disorder is a deficiency disease. It is because of the absence of the maturation principle that defective red cells enter the circulation and are then eliminated more rapidly than normal ones. Thus pernicious anemia is an example of a hemolytic syndrome caused by an intracorpuscular mechanism, namely a poorly constructed cyto-skeleton of the erythrocytes.

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THROMBOPENIC PURPURA; THE FAILURE OF DIRECT BLOOD TRANSFUSION TO RAISE THE PLATELET LEVEL*

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IT IS the purpose of this report to show that the platelet level of patients with thrombopenia cannot be elevated significantly for any period of time by means of massive direct transfusions of blood. For a long time it has been our feeling, as well as that of others, that blood transfusions do little in patients with thrombopenic purpura except to replace blood which has been lost by bleeding. However, following blood transfusion in the amounts ordinarily used, the dilution factor is so great that it has been impossible to determine accurately whether platelets in the transfused blood remain for any appreciable time in the circulation of the recipient.

Recently it was demonstrated in this laboratory that the circulating platelet level of the cat can be substantially raised for a period of a few days following cross circulation by way of carotid to cartoid anastomoses with a normal animal.[3] The technique, in effect, constituted a method of giving the thrombopenic recipient a massive direct blood transfusion through a continuous endothelial anastomosis. The experimental results made it seem pertinent to determine whether the platelet level in human subjects with very low platelet counts could be raised by massive direct transfusions of whole blood. On a priori grounds it seemed that massive direct transfusions should appreciably raise the platelet level, and that if the blood platelet in man has a normal rate of utilization of the same general order of magnitude as that found in the cat, this increased value should be demonstrable for a period as long as a few days. It was argued further that such an increase in the platelet level should have a favorable effect on hemostasis in thrombopenic individuals, and thus massive transfusions of whole blood would offer more benefit than the conventional indirect transfusion of 1 or 2 units of blood. Since the animal experiments had shown the life span of blood platelets to be much greater than that of the leucocytes under comparable conditions, it was hoped that thrombopenic purpura could be combatted by transfusions more effectively than agranulocytosis. Accordingly, massive direct blood transfusions were given to two patients with marked thrombopenia.

The medical literature records only two pertinent publications of which we are aware. Duke[1] in 1910 reported that direct blood transfusion both raised the platelet counts and improved the hemostasis of three patients with thrombo-

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penic purpura. In one case a rise in platelet count of 120,000 per cubic millimeter was noted. No data are given as to the method of direct transfusion employed or as to the amount of blood transfused. Krasso[2] in 1927 likewise recorded beneficial effects from blood transfusion in thrombopenic purpura. In one case, following the transfusion of a single unit of blood, the platelet count rose from 11,000 to 114,000 per cubic millimeter one day following transfusion. In the second case no significant elevation in platelet level occurred until six days after the transfusion of 450 c.c. of blood. The amounts of blood transfused and the time intervals concerned are such that it seems unlikely that survival of transfused platelets could per se account for the observed elevations in the platelet counts.

The failure of blood transfusion to replenish satisfactorily the circulating nonerythrocytic formed elements when these are dangerously low can be attributed to at least five possible causes. (1) The life span of these elements may be so short that when transfused they rapidly disappear merely in the course of normal utilization. This is true in the case of the white blood cells. (2) The elements may be qualitatively or quantitatively altered within a short period in stored blood. This factor is of undoubted importance. (3) The transfused elements are rapidly dispersed in the comparatively large blood volume of the recipient and, in many instances, are diluted to the level of insignificance on this basis alone. This is certainly true in instances where only 500 c.c. or less of blood are transfused. (4) The abnormal state which prompted the transfusion initially may result in an abnormally rapid rate of disappearance of the transfused elements. It is, of course, difficult to exclude this situation. (5) The recipient may destroy the donor elements more rapidly than his own. No satisfactory way of proving this exists.

CASE 1.—J. J., a 37-year-old Italian man, was first admitted to Strong Memorial Hospital in 1943 with complaints of dizziness, blurred vision, numbness and tingling of the left hand and left leg, and difficulty in walking. There was generalized pallor of the right optic disc. No objective sensory changes were observed. There was some ataxia in the finger-to-nose test with the left hand. The blood Wassermann was negative. The erythrocyte count was 5,820,000 per cubic millimeter; the hemoglobin was 15.8 Gm. per cent, and the leucocyte count was 10,900 per cubic millimeter with a normal differential. A diagnosis of multiple sclerosis was made and the patient was given a course of intravenous typhoid vaccine. The patient was able to continue working and except for episodes of transient numbness the symptoms improved.

In December 1945, the patient began to note pallor, exertional dyspnea, weakness, and fatigue. He was admitted to the Rochester General Hospital where he was found to have a severe anemia. Sternal marrow aspiration specimens suggested an aplastic bone marrow. The patient received numerous blood transfusions with but transient benefit and was referred to Strong Memorial Hospital on April 2, 1946, for further study. At the time of admission there were petechiae over most of the legs, and the patient's story indicated that a petechial rash had first appeared three months previously in the areas between the first and second fingers bilaterally. The frequent occurrence of small hematomas had been noted for two months before admission. There had been frequent free bleeding from the gums for two weeks prior to admission. No history of ingestion of drugs of the type which are known to predispose to aplastic anemia could be elicited.

On physical examination, with the exception of pallor of the optic discs the pertinent findings were limited to generalized pallor and the wide distribution of hemorrhagic mani-

festations. There were petechiae on the tongue and the mucous membrane of the mouth, oozing from the gums, clotted blood in the nostrils, and diffusely scattered petechiae over most of the body which assumed the proportion of hemorrhagic rash on the lower extremities. The spleen was not palpable.

At the time of admission the red blood cell count was 1,860,000 per cubic millimeter, the hemoglobin, 7.2 Gm. per cent, and the leucocyte count, 3,050 per cubic millimeter with but 29 per cent granulocytes. The urine showed 3 plus albumin and there was a positive test for blood. The stool showed a 3 plus guaiac test for blood. The clotting time (Lee-White method) was 9 minutes and clot retraction was poor, the clot being very friable and inelastic. The bleeding time was in excess of 42 minutes. The reticulocyte count was 1.8 per cent. The platelets were virtually absent from the blood film. A specimen of sternal marrow was removed surgically and a pathologic diagnosis of aplastic anemia was made. Despite indirect blood transfusion and vitamin K, the clinical course was progressively downhill. Continued bleeding was a troublesome problem and it was decided to attempt massive direct blood transfusion.

On April 5 the patient's blood picture was as follows, prior to transfusion: Erythrocytes, 1,505,000 per cubic millimeter; hemoglobin, 4.8 Gm. per cent; volume of packed cells, 13.0 per cent. The platelet counts on three separate determinations (Rees-Ecker method) were 4,000, 6,000, and 6,000 per cubic millimeter. The blood film was almost completely devoid of platelets. A direct transfusion of 690 c.c. was given and the patient developed a pyrogenic reaction necessitating discontinuation of the transfusion. Three hours after the transfusion the platelet count was 0 per cubic millimeter on two determinations. The following morning the erythrocyte count was 2,090,000 per cubic millimeter; the hemoglobin, 6.3 Gm. per cent, and the hematocrit, 17.0 per cent. The platelet count was 2,000 per cubic millimeter. In a two-hour period 1,500 c.c. of whole blood were transfused by the multiple syringe method. Despite this massive direct transfusion from normal donors, the platelet count two hours afterward was but 6,000 per cubic millimeter and there was no discernible elevation in numbers of platelets on careful examination of the blood film. Slow oozing of blood from the nose, present before the transfusion, never abated. The patient continued to bleed and died on April 13. No autopsy was performed.

CASE 2.—C. C., a 53-year-old white man, was admitted on Sept. 1, 1947, to the Eye Service complaining of a cataract of the right eye. The history indicated a hemorrhagic diathesis dating back to childhood with recurrent episodes of petechiae and easy bruising covering a period of many years. A splenectomy had been performed for idiopathic thrombopenic purpura in 1936. Following this there was only slight improvement in the bleeding tendency and the platelet count had remained depressed. However, in the year prior to admission there had been no significant episodes of bleeding.

The patient had noted failing vision since 1929 when he was found to have cataracts. In 1943 an iridencleisis had been performed following which the patient developed hemorrhagic glaucoma of the left eye. Since that time he had been virtually blind in the left eye, being able to discern only bright light and the vaguest outline of objects. Vision in the right eye was becoming progressively smoky and the patient was threatened with nearly complete blindness. Pertinent facts in the past history also included nephrolithiasis treated medically because of the bleeding tendency, hernia at the site of the previous splenectomy, and deafness of a mixed type in the right ear.

On physical examination the lens of the right eye was cloudy white and vision was greatly reduced. Vision was virtually absent in the left eye. There were surgical scars in the left upper quadrant of the abdomen and there was slight bulging in this area when the patient strained. There were a moderate number of scattered petechiae and a few larger purpuric areas. Physical examination was otherwise essentially normal.

The erythrocyte count was 5,700,000 per cubic millimeter, the hemoglobin, 18.5 Gm. per cent, and the leucocyte count, 10,850 per cubic millimeter with a normal differential formula. The platelet counts were 26,000, 27,000, and 28,000 per cubic millimeter on three determinations. The platelets were greatly reduced on the blood smear. The bleeding time was 5½

minutes on one occasion and 11½ minutes on another. The clotting time was within normal limits and the clot retracted somewhat. The blood fibrinogen was .328 Gm. per cent.

It was felt that removal of the cataract from the right eye was essential if the patient was to avoid blindness but that the hemorrhagic diathesis reduced materially the chances of successful operation. It was therefore decided to search for an accessory spleen prior to attempting to remove the cataract. In preparation for splenectomy the patient was twice phlebotomized and a total of 1,000 c.c. of blood withdrawn. He then received 1,500 c.c. of blood by direct transfusion from three normal donors. The Pennell apparatus[4] was employed and by this method it was possible to transfuse 500 c.c. of blood in eight minutes, assuring that the formed elements in the blood were a very short time outside the body. Unfortunately, technical difficulties with the platelet counting dilution fluid did not permit quantitative evaluation of the attempt to increase the blood platelets in this case. However, careful examination of blood films for platelets were made. It was felt that a very minimal increase in the numbers of blood platelets transiently occurred, but the increase was far below expectations and had disappeared completely within a few hours. The patient was operated upon the following day. There were many bleeding points but the patient did not bleed as much as was anticipated. No accessory splenic tissue could be found.

The only recourse left was to attempt to remove the cataract of the right eye in spite of the risk of hemorrhage. The alternative was blindness for the patient. Therefore a second attempt to prepare the patient was made using the same direct transfusion technique. As before, after phlebotomy, the patient received via the Pennell apparatus 1,500 c.c. of blood from three normal donors within a three-hour period. The day prior to transfusion the platelet counts were 18,000 and 23,000 per cubic millimeter and the bleeding time was 12 minutes. The morning of transfusion the platelet count on several determinations varied between 10,000 and 16,000 per cubic millimeter and the bleeding time from 10 to 17 minutes. Immediately after the transfusion the blood platelets had risen to between 50,000 and 80,000 per cubic millimeter; the bleeding time was 3 minutes. There appeared to be a moderate increase in the numbers of platelets on the stained films. However, two hours later the platelet count had fallen to 24,000 to 28,000 per cubic millimeter and the bleeding time was 5½ minutes. An hour later the platelets were below 20,000 per cubic millimeter. The morning following transfusion (which was the morning of the operation) the platelets were 20,000 per cubic millimeter and the bleeding time was 19 minutes. During the postoperative period the eyeball filled with blood, and at the time of this report, six months after operation, the patient was almost completely blind.

DISCUSSION

In each of the three transfusions reported, a minimum of 1,500 c.c. of whole blood was administered within a short period of time. If the blood volumes of the recipients were somewhere within the normal range, no more than three- to fourfold dilution of the transfused elements would be expected. If the blood platelets were received undamaged this would mean an elevation of at least 80,000 to 100,000 in the platelet count. On the basis of the experiments with animals, a detectable platelet increase persisting for an appreciable period of time would be anticipated. However, in only one of the three experiments reported was a significant increase noted in the recipient, and even in this case it was so ephemeral as to be of little practical value.

The reasons for the unsatisfactory results are not entirely clear. In Case 1 the patient (J. J.) had aplastic anemia, and, presumably, the fundamental defect to which the thrombopenia was due was merely a lack of platelet precursors. Though it cannot be said with certainty, there is no reason to believe that abnormally rapid platelet destruction was a factor in this case. Yet no elevation

in platelet count occurred and hemostasis as evidenced by slow oozing of blood from the nose remained unimproved. It was thought that the use of multiple glass syringes might have meant that few or no platelets were actually being reinjected into the recipient, but platelet counts on blood allowed to stand for periods of time up to five minutes in glass syringes indicated that large numbers of these elements were undoubtedly still present in the injected blood. However, it is quite possible that qualitative alterations in platelets during their brief stay outside the body may have resulted in their premature death on reinjection. Still another possibility is that the preceding thrombopenia created an abnormal need for platelets and that the injected platelets were almost immediately utilized for this reason. This seems unlikely. It is also possible that donor platelets are not utilized in a normal manner in human subjects, or that their normal rate of utilization is far greater than that found in animal experiments.

In Case 2 an attempt was made to obviate the effects on the platelets of being outside the body by using an apparatus which would reduce this period outside. The Pennell apparatus consists essentially of a short rubber tubing running between donor and recipient, the blood being milked from donor to recipient by means of a rotating worm. As much as 500 c.c. of blood were transferred from donor to recipient within eight minutes so that no unit of blood could have remained more than a very brief period outside the vascular system. Despite this, results were disappointing and the transient, slight elevation in platelet count was of no practical clinical significance. This patient (C. C.), of course, had idiopathic thrombopenic purpura, and it is not entirely clear whether or not platelet destruction takes place at a normal rate in this disease.

It should be emphasized that the direct transfusion techniques employed are by no means comparable to the continuous endothelial anastomosis of the animal experiments. Furthermore, in the animal work there was a much longer period of mixing of the blood of thrombopenic and normal animals and larger amounts of normal blood were mixed with thrombopenic blood. If comparable conditions had been present in the human subjects the results *might* have been different.

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A SIMPLE AND RAPID METHOD FOR DEMONSTRATING SICKLING OF THE RED BLOOD CELLS: THE USE OF REDUCING AGENTS

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INTRODUCTION

THE practical importance of a rapid and simple method of detecting the sickling phenomenon is apparent to anyone familiar with the various clinical manifestations of sickle cell disease. Unrecognized, this condition may masquerade as a variety of clinical entities, including rheumatic fever, osteomyelitis, cerebrovascular accidents, and abdominal conditions such as acute cholecystitis or appendicitis, presumably requiring prompt surgical intervention. Sicklemia may also be the cause of otherwise unexplained mild or severe anemia. Thus the ready performance of a simple test for sickling of the red cells may be useful in the hospital, in the physician's office, or even under field conditions in which genetic or anthropologic studies are being undertaken.

The sickling of the abnormal red blood cells capable of this remarkable alteration of the normal discoidal form is closely correlated with the concentration of reduced hemoglobin. The sickling phenomenon appears when the oxygen tension in the gas phase with which the blood is in equilibrium is 40 to 45 mm. of mercury or less.[1-3] Below this value the oxygen tension which causes manifest sickling depends on whether the active disease or only the so-called trait is present.[4] A fall in pH within the physiologic range increases the tendency to sickling at a given oxygen tension because of the resulting greater percentage of reduced hemoglobin in the red cells. When the red cells in a sample of blood are sickled, they form an interlacing network of crescentic, filamentous structures and as a result do not separate readily from the plasma under the influence of gravity or even of the centrifuge. Consequently when a sample of blood is sickled its sedimentation rate is decreased. Due entirely to the sickling of the red cells the viscosity[3] of the blood sample is also increased, as is the mechanical fragility[5, 6] of the red cells. These physical phenomena probably explain the thrombotic and hemolytic tendencies respectively which are so common in the clinical and pathologic manifestations of the disease.[3, 7, 8]

With the possible exception of the method recently proposed by Neuda and Rosen[9] which appears to depend on enzymic injury to the red cell surface, all clinical tests for sickling require the production of reduced hemoglobin. The resulting sickling is then judged by direct microscopic observation except when detected by the inhibitory effect of sickling on the sedimentation rate[10] in the

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tedious method of Winsor and Burch.[11] Once sickled, the red cells may be fixed in the sickled form with formalin solution.[4] Removal of oxygen from the red blood cells is effected either by physical means such as exposure of the blood to low barometric pressure (vacuum pump)[4] or at normal barometric pressure by displacement of the oxygen by equilibration of the blood sample with gases such as hydrogen, nitrous oxide, carbon dioxide, or nitrogen.[1-3] Oxygen and carbon monoxide cause prompt reversion to the discoidal form. A second principle employed is the reduction of the oxygen tension in the blood by denying access of more oxygen to the blood while that in the sample is being consumed metabolically. This may be carried out *in vivo* by causing stagnation of the circulation in a finger[2] for a few minutes with a tourniquet before withdrawal of a drop of capillary blood into formalin solution[4] or for rapid coverage beneath a coverslip. In vitro the same result may be achieved by allowing the consumption of the oxygen, chiefly by the white cells of the blood sample, to proceed in a sealed cover slip preparation.[12] The utilization of oxygen in such a system may be accelerated by incubation at body temperature or by the addition of leucocytes[4] or of aerobic bacteria.[4, 13] These methods are effective but do have the disadvantage of requiring special equipment such as vacuum pump, gas equilibration chamber, oxygen-free gases, stool filtrates, bacterial cultures or incubator.

METHOD

The simple method described here depends upon another principle, namely the reduction of the hemoglobin of the red cells by a chemical agent. Besides a drop of blood (which may be capillary blood taken directly from the ear or finger tip, or venous blood defibrinated or oxalated), only a microscope, microscopic slides and cover slips, and the reducing agent are required. Its only disadvantage is that because of the rapidity of its action it fails to distinguish the different susceptibilities to sickling of the red cells of active sickle cell disease and of the sickle cell trait. After trying several substances, ascorbic acid in the form of Cevalin* was chosen. It is readily available in the form of a buffered 10 per cent solution of the sodium salt with a pH of about 6.5 in sealed glass ampules containing 5 cubic centimeters. Because this particular solution when diluted with water to a concentration of 2.06 per cent was found to be approximately isotonic for red cells, a small amount of a 2 per cent solution is prepared, preferably from a freshly opened ampule, by fivefold dilution with water immediately before use.

The test has been in routine use in the Thorndike Memorial Laboratory of the Boston City Hospital for nearly two years and during this time has been taught to all Harvard second year medical students. The performance of the test is as follows. A *small* drop of the blood to be tested is placed upon a glass microscope slide and mixed with 1 or 2 drops of the diluted ascorbic acid solution. The mixture is at once covered with a glass cover slip on which pressure is momentarily exerted in order to extrude excess blood and produce a blood film sufficiently thin to permit satisfactory examination of individual red cells using the high-power dry objective of the microscope. The preparation is inspected for the presence of sickling at fifteen minute or other convenient intervals while standing at room temperature.

In originally determining the efficacy of the method, the cover slips were sealed at the edges with hot paraffin for additional protection against the entrance of oxygen. Control preparations were set up in a similar fashion except that either a drop of blood

*Kindly supplied by Mr. George B. Walden of Eli Lilly & Company, Indianapolis, Ind. According to Mr. Walden the composition of 100 c.c. of the solution, covered by a patent issued in 1940, is as follows: ascorbic acid, 11.0 Gm.; sodium carbonate, anhydrous powder, 3.25 Gm.; sodium metabisulfite, 0.55 gram.

alone or a drop of blood mixed with 1 or 2 drops of 0.85 per cent sodium chloride solution was sealed beneath the cover slip. In practice, only the preparation containing the ascorbic acid solution need be made, and the sealing of the cover slip with paraffin is unnecessary.

RESULTS

In Table I are shown the results of eighty tests for sickling made at various times on the blood of fourteen patients with sicklemia, including both anemic and nonanemic individuals. In 75 per cent of the samples, definite sickling appeared within one hour when the 2 per cent ascorbic acid solution was used as described. Sickling was present in all by the end of 3.5 hours at room temperature. On the contrary, in none of fifty control samples of blood from the same patients to which either nothing had been added or, in most instances, 2 drops of 0.85 per cent sodium chloride had been added did sickling appear within an hour. Indeed, sickling was present in only about 13 per cent of the control samples at the end of three hours, and after twenty hours 11 per cent of the control samples still failed to exhibit sickling.

TABLE I. EFFECT OF A BUFFERED 2 PER CENT SOLUTION OF ASCORBIC ACID CONTAINING 0.11 PER CENT SODIUM BISULFITE ON THE RATE OF FORMATION OF SICKLED CELLS IN SEALED WET COVER SLIP PREPARATIONS OF THE BLOOD OF FOURTEEN PATIENTS WITH SICKLEMIA

INTERVAL REQUIRED FOR DEFINITE SICKLING (HR.)	EIGHTY EXPERIMENTS		CONTROLS		
	POSITIVE TESTS		NUMBER OF CONTROLS	POSITIVE TESTS	
	NUMBER	PERCENTAGE		NUMBER	PERCENTAGE
0.25	9	11.3	52	0	0
0.5	27	22.5	51	0	0
0.75	36	43.8	50	0	0
1.0	60	75.0	50	0	0
1.5	66	82.5	43	2	4.4
2.0	77	96.2	37	2	5.4
2.5	78	97.5	32	2	6.3
3.0	79	98	30	4	13.3
3.5	80	100	28	4	14.3
5.0			24	7	29.2
10			15	11	73.3
20			46	41	89.1

The figures given are summations of the number or percentage of preparations exhibiting 5 to 20 per cent sickling at the interval stated. The time for the performance of the observations on the controls at five and ten hours occurred in many instances during the night and as a result an inspection was not made. Once the preparation containing ascorbic acid had become sickled, inspection of the controls tended to become less frequent. These factors led to the reduction in the number of controls observed in the interval between 1.5 and 20 hours as shown in the table.

DISCUSSION

That the effect of the ascorbic acid solution in causing sickling was in fact due to the reduction of the hemoglobin and not to some other action was proved by its inability to produce sickling in a hanging drop preparation, that is, one freely exposed to air. The reducing action of the ascorbic acid solution, however, for the purposes of the test described here, was shown to be independent of the rate of metabolic consumption of oxygen by the leucocytes in the preparation. Thus, high and low white blood cell counts respectively were artificially produced in different portions of a single sample of heparinized venous blood

from a patient with sicklemia. By centrifugalization of the original sample which contained 7,200 white cells per cubic millimeter, removal of the buffy coat and its addition to another portion of the sample, two portions were obtained which contained respectively 4,000 and 38,300 white cells per cubic millimeter. As was to be expected from the results of others[4] in similar experiments, sickling appeared in a sealed preparation of the blood with the high white blood cell count within forty-five minutes, but failed to appear in similar preparations of the original blood and of the blood with the low white blood cell count even after fifteen hours. On the other hand, when to a drop of each of the three samples of blood was added a drop of the 2 per cent ascorbic acid solution in a sealed cover slip preparation, sickling appeared within one hour in the blood with the low white blood cell count and was observed to appear only a few minutes earlier in the other two specimens.

That besides the times saved in its performance, the use of the ascorbic acid solution enhances the accuracy of the sickling test is suggested by the fact that about 10 per cent of the controls, as shown in Table I, still failed to show sickling after twenty hours at room temperature. That acceleration of the process of sickling, which usually results from the incubation of the specimen, may not avoid this difficulty is suggested by the recent observations of Shen, Fleming, and Castle[14] who found that the red cells of patients with sickle cell disease may fail to sickle if the blood has been incubated for twenty-four hours at 37.5° C. in the presence of oxygen. Thus in actual practice it appears possible that if sickling in the sealed preparation in the usual clinical test is sufficiently delayed, the red cells of the specimen may lose, more or less completely, the power to become sickled. Possibly an unusually slow consumption of oxygen in specimens of blood containing relatively few leucocytes may be responsible for the negative results among the control specimens. At any rate, occasionally it has been our experience in the past that sickling has failed to appear in sealed cover slip preparations even when they were kept in the incubator for twenty-four hours, whereas sickling was readily demonstrable in the fresh blood upon exposure to carbon dioxide gas.

Reducing agents such as methylene blue, potassium ferrocyanide, thioglycolate, and lactic and succinic acids were not as efficient as the buffered 2 per cent solution of ascorbic acid for the performance of the sickling test. That other reducing substances may be employed successfully was suggested to us at the completion of these studies by the report of da Silva[15] who states that a 2 per cent aqueous solution of sodium hydrosulfite caused rapid sickling in unsealed cover slip preparations of sicklemia blood. We found this particular compound, $Na_2S_2O_4$,* not very active. Sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$,† in 2 per cent solution caused no sickling at the end of fifteen hours. Sodium bisulfite, $Na_2S_2O_5$,* however, in 2 per cent solution in water caused sickling of the same blood in fifteen or thirty minutes.

*J. T. Baker Chemical Co., Phillipsburg, N. J.
†Merck and Company, Inc., Rahway, N. J.

Sodium bisulfite (metabisulfite), which is present in a concentration of 0.55 per cent as a stabilizer of the sodium ascorbate in Cevalin, undoubtedly adds to the reducing power of that solution. However, a 2 per cent solution of ascorbic acid brought to pH 6.5 with sodium carbonate and without added bisulfite produced sickling within an hour. A 0.11 per cent solution of sodium bisulfite in 0.85 per cent sodium chloride, which is the concentration of bisulfite in the fivefold dilution of the Cevalin, appeared to be about as active as the diluted Cevalin itself. The 2 per cent solution of sodium bisulfite was without question the most active of the substances tested. (See Addendum.)

The probability that solutions of any reducing agent may deteriorate on standing exposed to air suggests the desirability of using solutions freshly prepared from the salt. This is easily done with sodium bisulfite, but crystalline ascorbic acid proved to be entirely too acid and consequently injurious to the red cells without the addition of alkali. Even after opening the ampule, however, Cevalin appears to keep its reducing properties in the icebox for some days although its exact rate of deterioration has not been ascertained. Although not as rapid in its action as the 2 per cent aqueous solution of sodium bisulfite, it has the practical advantage of requiring only simple dilution rather than weighing in its preparation.

Because formalin is a reducing agent it was assumed that for this reason tissues from patients with sicklemia when fixed with this substance more clearly exhibit sickling of the erythrocytes than do tissues fixed with Zenker's fluid.[5] This property of formalin is, however, not the explanation since experiments in which either previously sickled or unsickled red cells were mixed anaerobically with 10 per cent formalin in 0.85 per cent sodium chloride solution showed that the formalin fixed the red cells in whichever phase they were exposed to it. It should be pointed out, however, that the presence of sickled red cells in the capillaries of formalin-fixed tissues, though valid evidence of the sickle cell trait, does not necessarily demonstrate to what extent sickling was present during life. Sufficient anoxia to cause extensive erythrocyte sickling may have occurred only as an agonal event or only after the circulation had ceased with death or at the time of the surgical removal of the tissue. In contrast to formalin, Zenker's fluid without added acetic acid when diluted to 10 per cent with 0.85 per cent solution of sodium chloride readily caused reversion of sickled red cells to more or less normal appearing discoidal forms. It is therefore probably because of this effect that the tissues of patients with sicklemia when fixed with Zenker's fluid may not show the characteristically shaped red cells.

SUMMARY

A simple and rapid method of producing sickling of the red blood cells in wet cover slip preparations of the blood of patients with sicklemia is described. The principle on which the test is based is the production of reduced hemoglobin in the red cells by the addition of a reducing agent. In order to perform the test, a drop of a fivefold aqueous dilution of Cevalin (approximating a 2 per cent solution of buffered ascorbic acid and also containing 0.11 per cent

sodium bisulfite) or a drop of 2 per cent sodium bisulfite, $Na_2S_2O_5$, is added to a small drop of the patient's blood on a glass microscope slide. After mixing, a cover slip is dropped on the preparation and excess blood is expressed by gentle pressure in order to produce a film of blood sufficiently thin to permit inspection of individual red cells under the high-power dry objective of the microscope. With the diluted Cevalin solution, sickling of the blood usually appeared within an hour, and with the 2 per cent bisulfite solution it was often present within fifteen minutes at room temperature.

Appropriate experiments demonstrated that the rate of sickling of the red blood cells caused by the ascorbic acid solution is, for the practical purposes of this test, unaffected by the rate of metabolic consumption of the oxygen by the white blood cells of the specimen. It was shown that formalin, although a reducing agent, promptly fixes the red blood cells in whichever form they are exposed to it, whether sickled or unsickled. Zenker's fluid, however, caused already sickled erythrocytes to reassume a discoidal appearance, which probably accounts for the fact that histologic preparations of tissues from patients with sicklemia when fixed with Zenker's fluid may not exhibit sickled red cells in the capillaries as clearly as when the tissues are fixed with formalin.

ADDENDUM

Since this article went to press, two communications have been reported. Dr. Janet Watson, of the Long Island College of Medicine, has reported by personal communication observations made by Dr. James McGovern, of Bellevue Hospital, New York. These investigators observed sickling in cover slip preparations, occurring in less than five minutes when using a 1 per cent solution of sodium acid sulfite (bisulfite) $NaHSO_3$-Baker. The first appearance of any sickled cells around the periphery of the cover slip preparations was considered as the end point.

Dr. Louis Thomas and Dr. Chandler A. Stetson, Jr., have recently made a preliminary report on "Sulfhydryl Compounds and the Sickling Phenomenon."* These authors report sickling in less than one-half hour in wet preparations made with reducing substances such as a saturated solution of hydrogen sulfide, cysteine (0.5 molar solution), and BAL (0.1 molar solution of 2,3-dimercaptopropanol).

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ANTI-A AND ANTI-B ISOAGGLUTININ TITERS IN Rh-IMMUNIZED PREGNANT WOMEN

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THE discovery of the ABO blood types, their heredity and antigenic properties, and their significance in blood transfusions brought forth many years ago the question whether ABO incompatibility during pregnancy might be responsible for disease during pregnancy or in the newborn infant. Very few convincing cases have been reported presenting conclusive evidence that ABO incompatibility may be of pathologic importance during pregnancy.[1, 2]

On the other hand, the antigenic properties of the A and B blood groups in pregnancies with ABO incompatibility have been demonstrated several times.

Jonsson[3] was the first to demonstrate the presence of potent anti-A and anti-B lysins in the mothers about three months after the delivery as a rather selective response to A or B antigen in the fetus.

Boorman and co-workers[4] and Smith[5] have confirmed this by studying isoagglutinin titers. They found only a slight increase during pregnancy, but a rapid and marked increase was seen shortly after delivery. Smith found this increase of titer only in cases where the infants were secretors.

The discovery of incompatibility in the Rh-system as the most common cause of hemolytic disease in the newborn infant gave rise to new problems such as: Why is Rh immunization during pregnancy not a more common occurrence in view of the fact that the possibility of Rh immunization so often exists? And another question: Is there any relation between Rh and A or B immunization?

With regard to the latter question Levine[6] has found a somewhat higher incidence of Rh immunization in ABO compatible pregnancies. Chown[7] and Davidsohn[8] found an increased AB incidence in Rh-immunized women. On the other hand, Gurevitch and co-workers[9] have published two cases where there apparently might have been a positive correlation between A and Rh immunization. In conformity with this, Wiener[10] has found in Rh-immunized women who have borne A or B children in ABO incompatible pregnancies a high anti-A or anti-B titer respectively.

This study, which is based upon blood samples from pregnant women, aims at an investigation of anti-A and anti-B isoagglutinin titers in Rh-immunized women compared with adequate control material. The results thus obtained necessitated an investigation of the various anti-Rh fractions. The work was carried out during the autumn of 1947.

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From the State Institute of Public Health, Serodiagnostic Department. Chief: Otto Hartmann.

MATERIAL AND METHODS

Material.—The anti-Rh-containing sera which were examined were samples obtained from women in the last two months of pregnancy. Of 25,000 samples 87 contained Rh antibody.

Methods for Routine Rh Determination.—The samples were tested for Rh in the usual manner. Cells from the coagula were suspended to 2 per cent in saline. One drop of the suspension was mixed in small test tubes with 1 drop of anti-Rh serum. The tubes were incubated at 37° C. for two hours, and the sediment then was examined for agglutination on slides under the microscope. Two different anti-Rh testing sera were used parallelly, one containing anti-D only and one containing anti-D + C, both possessing good agglutinating power in saline media. Sera from all samples, including Rh-positive ones, were tested by the same technique against a panel of Rh-genotyped O cells. Three drops of serum plus 1 drop of a 2 per cent suspension of cells in saline were used. Sera giving dubious reactions and sera from Rh-negative individuals to a great extent were examined also by Diamond's slide test or tested against cells suspended in serum and/or albumin and also partly tested by the Coombs' technique.

Storage and Control.—The Rh-containing sera were stored at −10° C., and groups of thirty sera then were tested. As a control for the isoagglutinins anti-A and anti-B, 100 sera from Rh-negative pregnant women who were not immunized and 100 sera from R-positive pregnant women were used. The control sera were picked out at random over a period of six months and were stored and handled in the same way as the sera containing Rh antibody.

Determination of the Various Anti-Rh Fractions Contained in Rh Sera.—The anti-D fraction contained in the anti-Rh sera was determined by titration both in serum and in saline media against O R_0r cells suspended in serum and in saline respectively. The serum media used were mixtures of fresh O Rh+ sera from blood donors. The titration was performed in relatively large Rh test tubes with a relatively heavy calibered capillary pipette graded to approximately 0.06 cubic centimeters. The same pipette was used for the distribution of dilution fluid and for the distribution of the suspension of cells. After incubation for two hours at 37° C. the test was read microscopically.

The sera were also tested against O R′r and O R″r cells to ascertain the content of anti-C and anti-E agglutinins. Two drops of the serum were mixed with 1 drop of the cells suspended in saline. All of the eighty-seven anti-Rh sera also were tested to ascertain the content of anti-C^w agglutinin, all sera being parallelly tested against O R_1^wr and O R_1r cells in which D-antigen was blocked. Blocking was performed with a serum containing anti-D + E exclusively in incomplete form (blocking serum taken post partum after the third stillbirth in an Rh-negative woman who was married to an R_2r or R_2R_2 man). One volume of washed, packed O R_1^wr and O R_1r cells was mixed with 50 volumes of a 25 per cent blocking serum in saline, incubated for fifteen minutes at 37° C., centrifugated and washed with saline once, and then suspended to 2 per cent in saline. One drop of this suspension was added to 2 drops of anti-Rh serum. Otherwise the usual technique was used.

Determination of Anti-A and Anti-B Isoaglutinins.—The anti-A and anti-B isoagglutinins in all three groups were then determined in parallel series against the same Rh-positive A_1 and B cells from fresh coagula. The cells were suspended to an approximately 1 per cent suspension in isotonic saline. The titrations were performed in successively doubled dilutions in test tubes. The volumes of each serum dilution and cell suspension were 0.2 cubic centimeter. The test tube racks were then placed overnight at +5° C. and afterwards for one hour at room temperature. The reading of end point titers was done macroscopically against a strongly illuminated white background, direct illumination on the tube was avoided.

RESULTS

For calculating the titer results the $\log_2$ of the conventional titer values was used (titer step values). The sera agglutinating cells in the first tube only

then have the titer value 1. In Tables I and II the arithmetic averages for the titer values in the various groups are recorded with the deviation of the means:

$$\mu = \sqrt{\frac{\Sigma (X-\overline{X})^2}{(n-1)\, n}}$$

Where X is the single observation in the group
Where $\overline{X}$ is the arithmetic average in the group
Where n is the number of observations in the group

Whether differences between the average values had any real, statistical importance was tested with the formula $t = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\mu_1^2 + \mu_2^2}}$ which is the difference divided by the deviation of the difference. That t is greater than 3 was taken as a sign that the difference is real (not due to chance).

Furthermore, some correlation coefficients (r) were calculated even though the material really was too small for such an analysis:

$$r = \frac{\frac{1}{n}\Sigma X \cdot U - \overline{X} \cdot \overline{U}}{\sigma X \cdot \sigma U} \quad \text{where} \quad \sigma X = \sqrt{\frac{\Sigma (X - \overline{X})^2}{n - 1}}$$

is the deviation of X in the group.

The Antibody Content in the Sera.—Prozone phenomenon against O R_0 r cells in saline medium was seen in five sera, that is no agglutination in the first or in the second and third tube, no prozone in serum medium. A relatively large number (four) of the sera contained anti-D+C+E. For three of these sera some kind of family control was obtained: Rh-negative multiparas married to men of the genotype C+, D+, E+, c+.

TABLE I. ANTI-D TITERS AND THE CONTENT OF ANTI-C, C^w, E IN ANTI-RH SERA AND THE DISTRIBUTION TO THE ABO SYSTEM

ABO TYPE	TYPE OF Rh ANTIBODY	NUMBER	AVERAGE OF ANTI-D TITERS					
			NaCl MEDIUM			SERUM MEDIUM		
A	Anti-D only	23	2.39±	0.50	t = 2.80	4.91±	0.74	
	Anti-D+C	9						
	Anti-D+C+C^w	6			t = 1.68			t = 1.74
	Anti-D +E	4						
	Anti-D+C +E	3	3.55±	0.47	t = 3.85	6.64±	0.65	
O	Anti-D only	15	2.53±	0.62	t = 1.66	4.13±	0.73	
	Anti-D+C	11						
	Anti-D+C+C^w	6			t = 1.64			t = 2.60
	Anti-D +E	1						
	Anti-D+C +E	1	3.89±	0.54	t = 3.19	6.89±	0.76	
B	Anti-D only	5	3.20±	1.28		3.60±	1.16	
AB	Anti-D only	2						
	Anti-D+C	1	1.0±	0.0		2.66±	1.66	

The distribution of the anti-Rh-containing sera to the ABO and MN system was within normal limits.

The effect of the serum medium on the anti-D titer values is so marked in the complex anti-Rh sera (those containing anti-D+ C, C^w, E) that the difference between the anti-D titer values in serum medium and saline medium is of statistical importance. The correlation between titer values for anti-D in serum medium and in saline medium is determined by the following correlation coefficients:

In A blood: $r_{\text{anti-D NaCl, anti-D serum}} = +0.34$

In O blood: $r_{\text{anti-D NaCl, anti-D serum}} = +0.60$

TABLE II. AVERAGE TITERS FOR ISOAGGLUTININS: ANTI-B AND ANTI-A

ABO TYPE	TITER	GROUP I Rh NEGATIVE WITH Rh ANTIBODIES	GROUP II Rh POSITIVE WITHOUT Rh ANTIBODIES	GROUP III Rh NEGATIVE WITHOUT Rh ANTIBODIES
A	Anti-B	6.06	7.12	7.51
	μ	0.25	0.15	0.18
	Number	45	51	49
O	Anti-B	6.41	7.40	7.73
	μ	0.22	0.15	0.19
	Number	34	40	41
	Anti-A	7.79	7.60	7.81
	μ	0.30	0.16	0.16
	Number	34	40	41
B	Anti-A	7.20	7.89	7.70
	μ	0.37	0.24	0.25
	Number	5	9	10

Table II shows that the anti-B titers in A and O blood in Rh-negative women with Rh antibody are considerably lower than in the control group, while the anti-A titers in O and B blood show no noticeable difference between the groups. The difference in anti-B titers was tested by the method previously outlined.

ANTI-B TITERS IN A BLOOD					ANTI-B TITERS IN O BLOOD				
Group	III	against	I	t 4.67	Group	III	against	I	t 4.55
Group	II	against	I	t 3.63	Group	II	against	I	t 3.72
Group	III	against	II	t 1.63	Group	III	against	II	t 1.38

Thus it appears that the low anti-B titers in Rh-negative women with Rh antibody compared with the control groups give differences which are too large to be caused by chance. A similar testing of the anti-A titers in O blood gave values less than 1. The low anti-B titers in Rh-negative, Rh-immunized women suggests that there may be an antagonism between anti-B and anti-Rh, and, furthermore, a possible negative correlation between the anti-B titers determined against B Rh+ cells and the anti-D titers determined against O R_o r cells. The correlation coefficient for the connection between anti-B titers and anti-D titers in saline medium, and between anti-B titers and anti-D titers in serum medium was calculated therefore, even though the material was too small for such an analysis.

	CORRELATION COEFFICIENTS (r) IN A BLOOD	CORRELATION COEFFICIENTS (r) IN O BLOOD
$r_{\text{anti-B, anti-D in NaCl medium}} =$	+ 0.43	+ 0.02
$r_{\text{anti-B, anti-D in serum medium}} =$	− 0.05	+ 0.11

That means that a simple negative correlation between anti-B and anti-D titers cannot be demonstrated.

COMMENT

It is possible that the average age of Rh-negative, Rh-immunized women is somewhat higher than that of the control group. It is a fact that the isoagglutinin titers decrease with increasing age after adult life. Titrating the anti-B in about 1,200 women of various ages, Hartmann[11] found a decrease of the average titer of approximately one-third titer step among women between 18 and 50 years of age. The anti-A titers were determined in the same way in about 700 women, and the decrease with age in titer values paralleled the decrease in anti-B titer. The anti-B titer in Rh-negative, Rh-immunized women is a whole titer step lower than the anti-B titers in the control group, while the corresponding anti-A titers show neither systematic nor definite statistical difference. A possible difference in age in the three groups does not, therefore, explain why the anti-B titer is so low in Rh-negative, Rh-immunized women.

SUMMARY

Eighty-seven antenatal anti-Rh sera were tested to ascertain the ABO-MN distribution and the content of various fractions of Rh anti-body and the anti-B and anti-A isoagglutinin titers.

The ABO and MN distribution was within normal limits. Lower anti-B titers are found in Rh-negative women with Rh antibodies compared with Rh-negative women without Rh antibodies and also compared with Rh-positive women. The difference (one titer step) is too large to be caused by chance. A possible negative correlation between anti-D and anti-B titers cannot be demonstrated.

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FACTORS IN THE USE OF MERCURIC BICHLORIDE FOR BIOLOGIC STUDIES

With Especial Reference to Blood Counts

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Hayem[1] first used the solution which has come to be designated by his name in order to meet his need of a diluent for experimental studies with blood. He simply adapted to his needs a fixative which he was accustomed to use for tissues in general. Its widespread adoption came about during the early phases of quantitative blood studies before demands for stringent accuracy had been formulated. Various workers have found inadequacies of distribution in its use and have offered formulas for other diluents as well as suggestions to lessen this difficulty with Hayem's solution. The errors in distribution in the use of the solution usually have been attributed to clumping or balling of cells in the diluting pipettes, though Jörgensen[2] also found unequal distribution on the counting chamber filled in the manner then in vogue. The clumping usually was ascribed to the age of the diluent and to a precipitate that may occur with age. Upon development of their mechanical rotor for mixing blood, Bryan and Garrey[3] found that while avoidance of these factors represents important precautions, it is safer to use a diluent for red blood counts that does not contain bichloride. They suggest the use of Toison's solution. They found the distribution of cells to be very exact when the rotor was used for white counts. Clumping does not occur in white counts. Ch'u and Forkner[4] also found that the presence of bichloride in the diluent causes clumping in certain pathologic blood, even when the pipettes are shaken by hand. They suggest that the clumping is due to the effects of the bichloride on the plasma proteins, but they did not investigate the problem from that angle and confined their studies to substitution of a different diluent, Gower's solution. These as well as other suggestions for substitution of different diluents never have received general adoption, and Hayem's solution has continued to be the diluent in common use without regard for the dangers of distribution inherent in its use. The causes underlying the inequalities of distribution in the use of bichloride for diluents were not investigated. Examination of the causes was, therefore, undertaken in an effort to so modify the formula of Hayem's solution as to avoid the dangers inherent in it, rather than to attempt further substitution of a different diluent.

It was found that satisfactory modification of Hayem's formula is possible from either of two approaches: (1) the content of bichloride can be reduced to as low as that in Jörgensen's solution, together with careful regulation of the pH

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within a narrow zone, or (2) a protective substance, such as gelatin or lecithin, can be added to the formula for Hayem's solution without consideration of pH up to 7.0. Use of gelatin was found to be the more practical method. The advantages of its use, preparation of the solution, and comparison between counts made with it and with Hayem's solution without gelatin or with Jörgensen's solution have been published briefly.[5] The studies that follow are those that were carried out to determine the causes that underlie the clumping of blood with diluents that contain bichloride and the factors that operate in the prevention afforded by the methods found. They are presented not only because of their pertinence to the use of diluents which contain mercuric bichloride for blood counts, but also because they indicate the significance of the pH and ionic content of diagnostic and therapeutic agents in the precipitative, agglutinative, and colloidal phenomena of the blood.

METHODS

The studies required approach from several aspects and it seems preferable therefore, on the whole, to describe the methods employed as each group of findings is presented. Consequently only those procedures will be described at this point which were used routinely to determine the data for each set of experiments.

All of the studies were made upon the blood of a single individual. Except where the studies were made on fractions of whole blood, all studies were made by drawing blood into automatic pipettes, both Trenner and Haak, from a freely flowing finger stick and immediately diluting with the desired solution. The pipettes were shaken a few times by hand and then rotated for thirty minutes or longer on a commercial model of the Bryan-Garrey rotor. Control studies made with one of the original rotors were entirely similar. The gentleness and smoothness of the movements of the rotor, together with the fact that it mixes in fixed planes, permit detection of very slight degrees of precipitation or aggregation, and therefore facilitated in the elucidation of the underlying factors that feature in the occurrence and prevention of clumping in blood counts.

The occurrence of precipitation, aggregation, clumping and so on in the pipettes was determined by microscopic inspection of counting chambers filled as for counts. Levy-Hausser chambers with improved Neubauer ruling were used.

The determinations of the hydrogen-ion concentrations of the solutions were made by means of a Hellige pH meter to which were attached the glass electrode and calomel tube of a Beckmann meter. Solutions of HCl and NaOH were used for adjustments of pH. Glass weighing bottles were used as containers for the solutions and the pipettes were filled directly from them immediately after adjustment. The fluid from the pipettes was, in turn, expressed directly into cups when the pH of the contents was to be determined.

PRESENTATION AND DISCUSSION OF EXPERIMENTAL DATA

The experimental data are presented in Tables I to IV and in Fig. 1. The values for pH stated in the following studies and recorded in the tables represent the pH of the diluents before they were mixed in the pipettes with the blood or fractions thereof. The pH readings of the resultant mixtures were modified according to the buffering action of the different biologic fluids, and curves are given in Fig. 1 to show the readings of the mixtures under various experimental conditions. The pH readings of the original diluents before experimental adjustments were as follows: Hayem's solution, 5.3; Hayem's solution plus gelatin, 4.9; Jörgensen's solution, 5.3; saline base, 6.0. All pH values

are recorded, approximately, to the nearest 0.5 reading. Blank recordings indicate lack of data at those values. Absence of reaction (that is precipitation, aggregation, or clumping) at any particular value is tabulated as zero. The presence of reaction at any pH reading is indicated by a plus sign. It does not seem important to indicate the degree or qualitative characteristics of the reaction since these are regulated, in large part, by combinations of factors such as the physical characteristics of any particular precipitation and its capacity to be rolled into balls and to catch cells, by the presene or absence of plasma or serum in any particular test of the series, by surface forces of the cells,

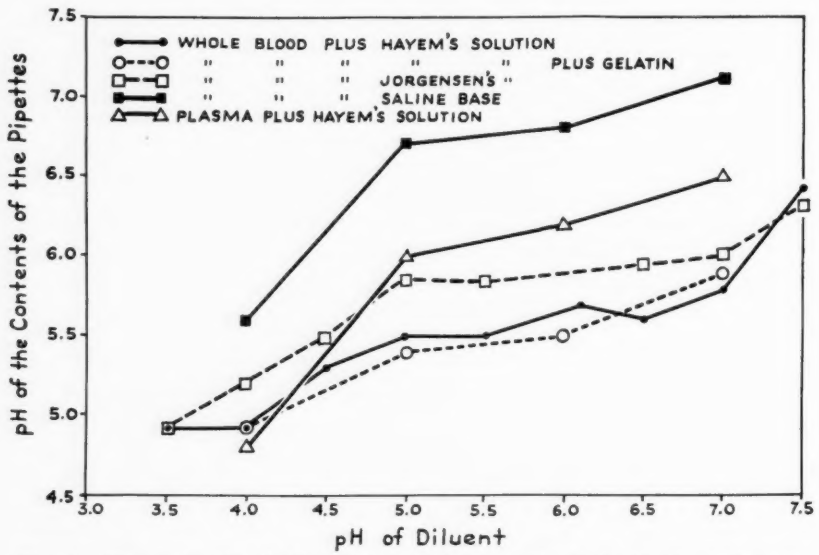

Fig. 1.—Change in pH of diluents by admixture with whole blood or plasma.

irrespective of precipitation within the plasma, and so forth. It is these factors which are often counteracted by vigorous shaking with the result that the underlying precipitation or aggregation is obscured. Therefore only the presence or absence of reaction in the pipettes at any given pH with any given diluent is tabulated, without record of the physical state of the reaction. The latter will be described and discussed where it seems significant to the understanding of the factors involved in its formation. Differentiation between certain terms used in these discussions may be aided by definition at this pont. The term precipitation is employed to indicate the occurrence of diffuse, fine, crystalline particles throughout the menstruum. The terms aggregation and agglutination are employed synonymously to indicate close adherence of erythrocytes to each other without adhesive mechanism that is microscopically visible. The two states differ, apparently, only in the number of cells adherent to each other. Agglutination apparently represents aggregation of sufficient cells to cause settling of the masses. The terms clumping and balling are used synonymously to indicate the conditions usually met in whole blood when cells and precipitate unite in fibrinous masses.

Action of Bichloride as a Fixative.—Studies made by the addition of other unrelated tissue fixatives to the saline base of Hayem's or Jörgensen's solution indicate that the clumping effect on blood of diluents that contain bichloride (Fig. 2, *3* to *6*) is related to its action as a biologic fixative. The solutions to be tested were mixed with whole blood in pipettes and rotated for a short period in the manner used in making blood counts with Hayem's or Jörgensen's solution. The saline base alone caused no clumping and only questionable lysis at the hydrogen-ion concentrations under study (Table I). Addition of absolute alcohol to the saline base in dilutions up to 90 per cent by volume caused lysis; above that it caused clumping similar to the clumping observed with Hayem's solution. The same statements hold for solutions of saline base and acetone. They caused lysis in dilutions up to 55 volumes per cent and clumping above that proportion. Formalin caused lysis when the pH was below 5.0. Above that, formalin caused neither lysis nor clumping in concentrations between 10 and 60 volumes per cent. Concentrations above that caused clumping. In similar manner a balance was found to exist between hemolysis

TABLE I. EFFECT OF PH ON CLUMPING OF WHOLE BLOOD WITH DIFFERENT DILUENTS

DILUENT	pH										
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Jörgensen's with 25% $HgCl_2$*					L	L	L	L	L		
					0	0	0	0	+	+	+
With 50% $HgCl_2$					L	L	L	L			
					+	+	+	+	+	+	+
With 75% $HgCl_2$			L	L	L						
			+	+	+	0	0	0	+	+	
With 100% $HgCl_2$			L	L							
			+	+	0	0	0	0	+		
With 125% $HgCl_2$		L	L								
		+	+	0	0	0	0	+	+		
With 150% $HgCl_2$			+	+	0	0	0	0	+		
With 100% $HgCl_2$ plus gelatin		0	0	0	0	0		0	0	+	+
Hayem's		+		+	+	+			+		
Plus gelatin	0	0	0	0	0	0	0	+	+		
Saline base		L?									
		0	0	0		0	0		0		0
Plus gelatin		0	0						0		0
Saline base plus 2 drops Na2WO4†	+	+		0	0				0		0
Plus 5 drops Na2WO4	+			+						+	
Plus 10 drops Na2WO4	+	+		+		+	+	+	+	+	+
Plus 10 drops Na2WO4 plus gelatin		+		+	+	+	0	0	0		

+, Positive reaction in the form of precipitation, aggregation, agglutination, or clumping; ?, opalescence, but no frank precipitate; L, some degree of lysis; 0, no visible reaction; blank, no studies made.

*The percentages of $HgCl_2$ recorded with the dilutions of Jörgensen's solution refer to the amount of $HgCl_2$ used compared with the normal amout for Jörgensen's solution.

†All solutions of NaWO4 were made by use of the same dropper and a 10 per cent stock solution.

and clumping, dependent upon the proportions of bichloride added to the saline base (Table I). When added in the proportion of 25 per cent of the amount in Jörgensen's solution (0.012 Gm. to 100 c.c.) lysis alone occurred below pH 7.0, and clumping above. In a proportion of 50 per cent of that in Jörgensen's solution both lysis and clumping occurred when the pH was below 7.0, and clumping only at higher values. When the content was 75 per cent of that in Jörgensen's solution lysis accompanied by clumping occurred only below pH 6.0, while clumping alone occurred above pH 7.0. The narrow zone between these values was neutral as far as these effects were concerned—neither lysis nor clumping occurred. With full strength Jörgensen's solution, that is 0.05 Gm. per 100 c.c., the neutral zone was extended somewhat on the acid side but otherwise the events were similar to those with 50 per cent concentration. When the concentration was 125 per cent of that in Jörgensen's solution the neutral zone was extended still more to the acid side, while the events in the basic direction remained unaltered. At a concentration of 150 per cent of that in Jörgensen's solution, lysis ceased and clumping alone occurred on both sides of a neutral zone, and the reactions became more like those obtained with Hayem's solution. With the latter, lysis did not occur at the pH values tested, but clumping occurred at all pH levels, with a tendency, however, to a neutral zone in the same region as the neutral zones with the weaker solutions.

It seems obvious therefore that many, if not all, fixatives function in two ways. If sufficiently dilute they cause more or less lysis, which may or may not be accompanied by clumping of cells that have not been destroyed; the liability to lysis is influenced not only by dilution but also by the pH of the diluent. If sufficiently concentrated the fixatives no longer cause lysis but exert only a clumping action. Experimental explanation of these actions, as far as bichloride is concerned, is presented in the groups of studies that follow.

The danger of lysis as well as of clumping in the use of Jörgensen's solution as a diluent for red counts, unless the pH is carefully controlled, is obvious. The clinical significance of this is discussed elsewhere.[5]

The Relationship of the pH of the Diluent to Clumping in Whole Blood (Table I).—As stated previously, addition of lecithin or gelatin was found to prevent the clumping inherent in the use of Hayem's solution (Fig. 2, compare *2* and *7* with *1*, *3*, *4*, *5*, and *6*). The factors involved in this protective action

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Fig. 2.—The low-power magnifications were obtained with a 16 mm. objective and a ×15 ocular; the high-power magnifications, with a 4 mm. objective and ×10 ocular. The diluting pipettes were rotated for thirty minutes, beginning immediately after filling. *1*, Illustrates about the best distribution obtainable with standard, unadjusted Hayem's solution. Even in this case the tendency toward aggregation of cells is demonstrable in the form of tiny groups scattered here and there. The pH of the Hayem's solution was 4.8. *2*, Distribution with Hayem's solution containing gelatin. The mixture had been left in a Pyrex flask for many months. Aggregation of cells did not occur. The pH of the mixture was 4.6. *3*, Detail of the aggregates in preparation made with Hayem's solution adjusted to pH 3.1. Small masses of cells aggregate rather tightly. Little precipitate occurs in the plasma and, if present, it rarely becomes intermingled with the aggregates of cells. *4*, Same solution as *1* but from a different pipette. The tendency toward aggregation of cells is more pronounced in this preparation. *5*, Same as *4*. Detail of the aggregates in preparations made with standard, unadjusted Hayem's solution. Only a few cells occur in each aggregate and they are loosely balled with a soft amorphous precipitate. *6*, Detail of the aggregates in preparations made with Hayem's solution adjusted to pH 7.2. Cells agglutinate into large tight masses and these become enmeshed with large amounts of refractive, fibrinous precipitate. *7*, Same as *2*. Detail of preparations made with Hayem's solution containing gelatin. There is no tendency toward aggregation of cells and any precipitate remains scattered.

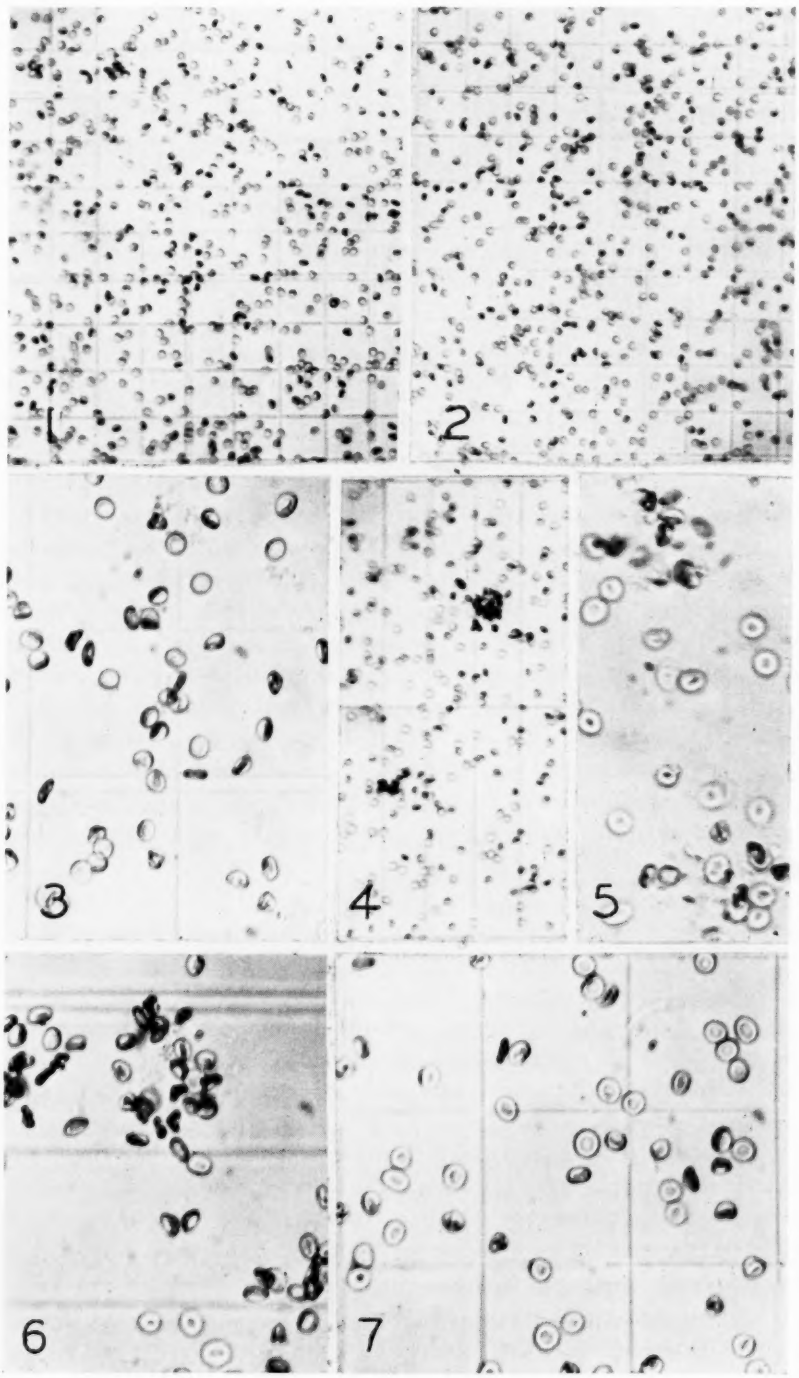

Fig. 2.

(See opposite page for legend.)

have been studied in detail for solutions containing gelatin. The gelatin was added in the proportion of 0.01 Gm. to 100 c.c. of Hayem's solution. The method of preparation and the precautions have been published.[5]

It already has been shown that Hayem's solution caused clumping of the blood at all pH levels that were studied. It may be seen from Table I that when the solution contained gelatin, however, clumping was inhibited below pH 7.0. If the proportion of bichloride in the diluent was reduced to that in Jörgensen's solution (as was also discussed in the preceding section) clumping occurred on both sides of a neutral zone, between pH 5.0 and 7.0, in which neither clumping nor lysis occurred. In addition to clumping, lysis of some of the cells occurred on the acid side of that zone. As the concentrations of bichloride were reduced, lysis and clumping on the acid side of the neutral zone took place at increasingly greater pH, with consequent narrowing of the zone. As the concentrations of bichloride were increased, lysis and clumping on the acid side of the neutral zone occurred only at increasingly lower pH, with consequent widening of the zone. When the concentration was sufficient, lysis did not occur at all and the reaction became similar to that with Hayem's solution. It may be seen from Table I that gelatin afforded the same protective action against clumping with the weaker solutions of bichloride that it did with Hayem's solution. In addition it also protected against the lysis caused by weak acid solutions of bichloride. The studies with these weaker solutions reveal, therefore, something of the character of the clumping effect of bichloride better than those made with a concentration as great as that in Hayem's solution. They bring out the fact that clumping with bichloride is more intense on both sides of a middle zone of pH and that when the solution is weak enough a neutral zone at which clumping does not occur becomes definite. Addition of gelatin is effective only on the acid side of that zone.

In addition to the preceding findings with the solutions of bichloride, there also were observed qualitative, though not necessarily quantitative, differences in the character of the clumping at different levels of pH. At very low values the erythrocytes aggregated into groups that seemed free of any surrounding material (Fig. 2, *3*). At higher values they also often were aggregated, but in addition they were always enmeshed or embedded in an amorphous or fibrinous background (Fig. 2, *5* and *6*). This background varied in appearance from a faintly amorphous character at the lower pH readings to a coarse, refractive, fibrinous nature at the higher readings. The more fibrinous the appearance, the more scattered were the cells in and about the background so that the clumps took on the appearance which is commonly observed when clotting has been allowed to occur before addition of the diluent.

When sodium tungstate was substituted for bichloride in the saline base and this diluent was used with whole blood in the same manner as the solutions containing bichloride, clumping occurred at all pH values when the metallic salt was concentrated, but only at pH 4.0 or below when it was weak (Table I). Similar to the reaction in solutions containing bichloride, addition of gelatin to the diluent prevented clumping in one direction of pH, but in contrast to its

effect in solutions containing bichloride, it prevented clumping in the basic instead of the acid direction. In other words, strong and weak concentrations of sodium tungstate exerted an effect on whole blood analogous to that of bichloride, except that the pH level at which clumping occurred, irrespective of the presence of gelatin, was reversed to the acid side. In addition, lysis was not detected under any of the experimental circumstances. These reactions between whole blood and sodium tungstate will be discussed further under Correlation and Discussion of Experimental Findings.

In summary, these studies reveal the following facts: first, with concentrated solutions containing bichloride clumping of cells occurred at all pH levels, but the character of the clumping varied; second, with weaker solutions of bichloride a neutral zone was present at which clumping did not occur, but variable degrees of lysis, as well as clumping, occurred on the acid side of that zone and clumping only on the basic side; third, solutions of sodium tungstate, instead of bichoride, acted in a manner similar to solutions of bichloride except for reversal of the critical level of pH; clumping occurred at all levels with concentrated solutions, but on the acid side only with weak solutions; fourth, gelatin gave protection from the clumping of blood with solutions of mercuric bichloride below pH 7.0 or sodium tungstate above pH 6.0. It also protected the cells from lysis with weak acid solutions of bichloride.

It is obvious that clumping of the blood is associated with the presence of metallic salts and is prevented by the addition of gelatin at certain ranges of pH, the range varying with the salt employed. The following studies were carried out to determine the part played by the different fractions of the blood in the clumping and the nature of the forces that respond to pH and of those that are inhibited by gelatin.

Studies With Whole Washed Erythrocytes (Table II).—Blood for these studies was collected by venous puncture in tubes containing either dried sodium citrate or 1 drop of a concentrated solution of sodium oxalate. The tubes were centrifuged and the plasma was collected separately for studies to be described later. The cells were washed with about 10 volumes of physiologic saline, centrifuged, separated, and rewashed until the process had been repeated six or more times. The erythrocytes were then suspended in equal volumes of saline and the suspension was used with the pipettes and various diluents in the manner and under the conditions described in the studies with whole blood. These plasma-free cells were found to aggregate, or agglutinate, not only with Hayem's solution but also with all dilutions of Jörgensen's solution employed, almost irrespective of the pH. The aggregation was similar to that observed with whole blood in Hayem's solution at very low pH values (Fig. 2, *3*). That is, the aggregates appeared free of any amorphous or fibrinous background. The aggregation was inhibited by gelatin below pH 6.5. With sodium tungstate, on the other hand, aggregation of erythrocytes occurred only on the acid side and was unaffected by gelatin.

It is obvious from these findings that while the fibrinous qualities in the clumping of whole blood with bichloride are related to the presence of plasma, aggregation of the erythrocytes is independent of the plasma. Furthermore,

have been studied in detail for solutions containing gelatin. The gelatin was added in the proportion of 0.01 Gm. to 100 c.c. of Hayem's solution. The method of preparation and the precautions have been published.[5]

It already has been shown that Hayem's solution caused clumping of the blood at all pH levels that were studied. It may be seen from Table I that when the solution contained gelatin, however, clumping was inhibited below pH 7.0. If the proportion of bichloride in the diluent was reduced to that in Jörgensen's solution (as was also discussed in the preceding section) clumping occurred on both sides of a neutral zone, between pH 5.0 and 7.0, in which neither clumping nor lysis occurred. In addition to clumping, lysis of some of the cells occurred on the acid side of that zone. As the concentrations of bichloride were reduced, lysis and clumping on the acid side of the neutral zone took place at increasingly greater pH, with consequent narrowing of the zone. As the concentrations of bichloride were increased, lysis and clumping on the acid side of the neutral zone occurred only at increasingly lower pH, with consequent widening of the zone. When the concentration was sufficient, lysis did not occur at all and the reaction became similar to that with Hayem's solution. It may be seen from Table I that gelatin afforded the same protective action against clumping with the weaker solutions of bichloride that it did with Hayem's solution. In addition it also protected against the lysis caused by weak acid solutions of bichloride. The studies with these weaker solutions reveal, therefore, something of the character of the clumping effect of bichloride better than those made with a concentration as great as that in Hayem's solution. They bring out the fact that clumping with bichloride is more intense on both sides of a middle zone of pH and that when the solution is weak enough a neutral zone at which clumping does not occur becomes definite. Addition of gelatin is effective only on the acid side of that zone.

In addition to the preceding findings with the solutions of bichloride, there also were observed qualitative, though not necessarily quantitative, differences in the character of the clumping at different levels of pH. At very low values the erythrocytes aggregated into groups that seemed free of any surrounding material (Fig. 2, *3*). At higher values they also often were aggregated, but in addition they were always enmeshed or embedded in an amorphous or fibrinous background (Fig. 2, *5* and *6*). This background varied in appearance from a faintly amorphous character at the lower pH readings to a coarse, refractive, fibrinous nature at the higher readings. The more fibrinous the appearance, the more scattered were the cells in and about the background so that the clumps took on the appearance which is commonly observed when clotting has been allowed to occur before addition of the diluent.

When sodium tungstate was substituted for bichloride in the saline base and this diluent was used with whole blood in the same manner as the solutions containing bichloride, clumping occurred at all pH values when the metallic salt was concentrated, but only at pH 4.0 or below when it was weak (Table I). Similar to the reaction in solutions containing bichloride, addition of gelatin to the diluent prevented clumping in one direction of pH, but in contrast to its

effect in solutions containing bichloride, it prevented clumping in the basic instead of the acid direction. In other words, strong and weak concentrations of sodium tungstate exerted an effect on whole blood analogous to that of bichloride, except that the pH level at which clumping occurred, irrespective of the presence of gelatin, was reversed to the acid side. In addition, lysis was not detected under any of the experimental circumstances. These reactions between whole blood and sodium tungstate will be discussed further under Correlation and Discussion of Experimental Findings.

In summary, these studies reveal the following facts: first, with concentrated solutions containing bichloride clumping of cells occurred at all pH levels, but the character of the clumping varied; second, with weaker solutions of bichloride a neutral zone was present at which clumping did not occur, but variable degrees of lysis, as well as clumping, occurred on the acid side of that zone and clumping only on the basic side; third, solutions of sodium tungstate, instead of bichoride, acted in a manner similar to solutions of bichloride except for reversal of the critical level of pH; clumping occurred at all levels with concentrated solutions, but on the acid side only with weak solutions; fourth, gelatin gave protection from the clumping of blood with solutions of mercuric bichloride below pH 7.0 or sodium tungstate above pH 6.0. It also protected the cells from lysis with weak acid solutions of bichloride.

It is obvious that clumping of the blood is associated with the presence of metallic salts and is prevented by the addition of gelatin at certain ranges of pH, the range varying with the salt employed. The following studies were carried out to determine the part played by the different fractions of the blood in the clumping and the nature of the forces that respond to pH and of those that are inhibited by gelatin.

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It is obvious from these findings that while the fibrinous qualities in the clumping of whole blood with bichloride are related to the presence of plasma, aggregation of the erythrocytes is independent of the plasma. Furthermore,

it is obvious that plasma actually gives protection from aggregation in the neutral zone when the metallic salt is weak enough. Gelatin acts as a more potent protective agent and protects even with strong solutions of the metallic salt.

Lysis did not occur with the weak solutions of bichloride as it did with whole blood. It seems probable that the more fragile cells had been lysed automatically in the process of washing and that the remaining cells were sufficiently resistant for the conditions of the experiment.

Studies With Lysed, Washed, Erythrocytes (Table II).—The saline suspensions of erythrocytes that were used for the preceding studies with whole, washed erythrocytes were centrifuged and freed of as much saline as possible. They were lysed by the addition of about twice their volume of distilled water. The solutions were centrifuged and the supernatant fluid was filtered through

TABLE II. EFFECT OF pH ON CLUMPING OF WASHED RED BLOOD CELLS AND FILTERED LYSED RED BLOOD CELLS WITH DIFFERENT DILUENTS

DILUENT	pH										
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Washed Red Blood Cells											
Jörgensen's with 25% $HgCl_2$*		0				+				+	
With 100% $HgCl_2$ gelatin		0				0				0	
With 100% $HgCl_2$		+		+		+		+		+	
With 100% $HgCl_2$ plus gelatin		0		0		0		0	+		
Hayem's	+	+			+	+				+	
Plus gelatin	0	0			0	0	+	+	+		
Saline base plus 5 drops Na2W04†		+						0	0		
Plus 5 drops Na2W04 plus gelatin		+						0			
Saline base									0		
Lysed Red Blood Cells‡											
Jörgensen's with 25% $HgCl_2$		+				+				+	
With 100% $HgCl_2$		+	+	+		+	+	+	+	+	+
Drawn to automatic stop only		?				+					
With 100% $HgCl_2$ plus gelatin		?		?		?		+	+		
Hayem's	+	+	+			+		+			
Plus gelatin	+			+				+			
Saline base plus 5 drops Na2W04		+	+	0		0					0
Saline base		0				0					0

+, Positive reaction in the form of precipitation, aggregation, agglutination, or clumping; ?, opalescence, but no frank precipitate; 0, no visible reaction; blank, no studies made.

*See footnote * Table I.

†See footnote † Table I.

‡The fluid for these tests was drawn to about one-tenth the volume of the pipette instead of to the automatic stop.

No. 5 Whatman paper. No membranes could be found by microscopic examination after this treatment. The filtrate was subjected to tests similar to those employed with whole erythrocytes. If sufficient of the filtrate was used, it was precipitated by either Hayem's or Jörgensen's solution at all hydrogen-ion concentrations studied, and the precipitation was not prevented by the presence of gelatin in the diluent in the usual proportions. With sodium tungstate, precipitation occurred up to but not beyond pH 4.5. There was no difference between the reactions of the erythrocytes that had been procured by the use of oxalate and those procured by the use of citrate.

The unprotected, washed erythrocytes obviously had reacted to the diluents in accord with the reactions of their contents to the same diluents, but they could be prevented from certain of the reactions by the presence of gelatin, while the contents were not thus protected from reaction. It would seem, therefore, that gelatin must function by protecting the surface of the erythrocytes from contact with the metallic salt, but not beyond certain critical pH values. These values vary according to the nature of the metallic salt.

Studies With Plasma and Serum.—The plasma and serum for these studies were obtained at the same time as the cells used in the preceding studies. The plasma used was from the oxalated specimen only. These fluids were subjected to tests similar to those made on blood and cells. As in the case of the contents of lysed erythrocytes, if sufficient plasma was used it was precipitated at all hydrogen-ion concentrations tested by either Hayem'a or Jörgensen's solution and this action was not prevented by the presence of gelatin in the usual proportion. In order to simulate the conditions that exist in making blood

TABLE III. EFFECT OF pH ON PRECIPITATION OF PLASMA OR SERUM WITH DIFFERENT DILUENTS

DILUENT	pH										
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Plasma											
Jörgensen's with 25% $HgCl_2$*		0				0			0		
With 100% $HgCl_2$	0	0	0	+	+	+			+		
Hayem's		0				+			+		
Plus gelatin		0				+			+		
Saline base plus 5 drops Na2W04†		+				+				0	
Plus 5 drops Na2W04 plus gelatin		+				0			0		
Saline base	0					0				0	
Serum											
Jörgensen's with 25% $HgCl_2$		0				0			0		
With 100% $HgCl_2$	0	0	0	0	0	0	+	+	+		
Hayem's		0				+			+		
Plus gelatin		0				+				+	
Saline base plus 5 drops Na2W04		0				0				0	

+, Positive reaction in the form of precipitation, aggregation, agglutination, or clumping; 0, no visible reaction; blank, no studies made.

*See footnote * Table I.

†See footnote † Table I.

counts, therefore, studies were also carried out on plasma drawn only to the automatic stop of the pipette (Table III). Under those circumstances it was not precipitated below pH 5.0 by the solutions of bichloride or above pH 6.0 by those of sodium tungstate. As was the case when larger amounts of plasma were employed, these precipitations with lesser amounts also were not prevented by the presence of gelatin in the usual proportions.

The findings with serum varied from those with plasma in two respects: the serum was not precipitated as easily nor at as low a pH with the weak solutions of bichloride as was the plasma, and it was not precipitated at all by the amounts of sodium tungstate that caused precipitation of the plasma.

CORRELATION AND DISCUSSION OF EXPERIMENTAL FINDINGS

With amounts equivalent to those present in blood counts, plasma was not precipitated by Hayem's solution below the isoelectric point of any of the usual plasma proteins. Precipitation did occur above that point, but the precipitation was at first soft and slight and fluffy, and it was only as the pH rose to levels above the isoelectric point of most of the common plasma proteins that precipitation became sturdy and abundant enough to ball with rotation. The same statements may be made of the reactions to Jörgensen's solution. Gelatin did not affect these precipitations. Yet gelatin prevented clumping in whole blood at hydrogen-ion concentrations equal to those at which precipitation of plasma proteins occurred. Obviously the clumping in whole blood is due to factors other than just the action of the metallic salts on the plasma proteins alone.

The washed erythrocytes aggregated, or agglutinated, at all pH levels with any dilution of the mercurial, and this could be prevented with gelatin up to pH 6.5. Yet if weak solutions of the mercurial were used with whole blood, clumping did not occur in a neutral zone. Therefore the plasma proteins gave protection from aggregation despite the fact that they themselves were not prevented from precipitation at this hydrogen-ion concentration. Again, obviously, the plasma proteins are not the entire cause of clumping in whole blood, and they are even somewhat protective, though less so than gelatin.

Irrespective of the presence of the plasma proteins then, clumping occurs in whole blood at all pH levels with concentrated bichloride solutions, and on either side of a neutral zone with dilute solutions. The clumping with both the weak and strong solutions of bichloride is prevented up to a certain critical pH of 7.0 by the addition of gelatin. Since the washed erythrocytes aggregated, or agglutinated, at all hydrogen-ion concentrations, even with the dilute solutions of bichloride, and the aggregations could be prevented up to pH 6.5 by the addition of gelatin, it seems obvious that the factors which cause aggregation of erythrocytes in the presence of the metallic salt also regulate the clumping in whole blood with this salt.

The contents of the lysed erythrocytes precipitated at all pH levels and this was not prevented by gelatin. The contents were obviously subject to reactions similar to those which characterize plasma proteins. Without gelatin the washed erythrocytes responded much as their contents. Yet gelatin protected them from aggregation up to pH 6.5. It is obvious that intact erythrocytes

exert a somewhat different or more concentrated force than that of free proteins, and it is this which can be prevented up to a certain point by the protective agent, gelatin. They apparently function, when whole, as large electronegative colloids which aggregate in the presence of positive ions.

Granted that they do function as electronegative colloids, the aggregation of erythrocytes in whole blood with weak solutions of bichloride on the far acid side of a neutral zone in which aggregation did not occur can be explained on the basis of colloidal aggregation due to the presence of sufficient positive electrolytes.

The occurrence of agglutination on the basic side of the neutral zone, in contrast to that on the acid side, may be due to the fact that the gelatin itself is precipitated at this pH (Table IV). On the other hand it may be due to direct reaction between the bichloride and the contents of the erythrocytes at a pH concentration above the isoelectric point of hemoglobin. Gelatin did not prevent precipitation of plasma proteins at pH concentrations above their isoelectric points, even when those concentrations were less than the pH at which gelatin could be inactivated by precipitation. It seems probable, therefore, that the aggregation of erythrocytes at pH 7.0 in whole blood, or 6.5 with washed cells, was due to the inherent character of the cellular contents rather than to the precipitation of the gelatin. The studies with sodium tungstate and washed erythrocytes seem to support these conclusions. Colloidal aggregation above a pH at which direct union could occur between the metallic radical and the proteins within the cells was apparently suppressed by the presence of negative electrolytes. The explanation of the capacity of concentrated solutions of sodium tungstate to cause clumping in whole blood at high pH concentrations and of the protection afforded by gelatin, in contrast to this reaction with washed erythrocytes, is not clear.

Whenever aggregation of erythrocytes occurs at pH levels at which precipitation of the plasma proteins may also take place, the aggregation is accompanied by a background of more or less amorphous or fibrinous material. It would seem that while the precipitated plasma proteins are not in themselves sufficient to initiate clumping, they do qualify and intensify it if aggregation

TABLE IV. EFFECT OF pH ON THE DILUENTS ALONE

DILUENT	pH										
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Saline base plus gelatin	0	0		0		0	0		0		
Saline base plus gelatin plus Na_2WO_4*			+	+	0						
Saline base plus gelatin plus Jörgensen's solution	0		0			0		0	0		
Saline base plus gelatin plus Hayem's solution							0	+	+		
Jörgensen	0	0		0		0		0	0		+
Heyem							0	+	+	+	+

+, Positive reaction in the form of precipitation, aggregation, agglutination, or clumping; 0, no visible reaction; blank, no studies made.

*See footnote † Table I.

or agglutination of the erythrocytes occurs. It is by this inhibition from aggregation that more vigorous shaking or the presence of gelatin acts to prevent clumping until critical pH levels occur.

That the clumping with weak, dilute solutions of bichloride at pH levels below those at which the plasma proteins precipitate was, nonetheless, accompanied by a fibrinous background is probably due to the lysis that occurred at those levels with liberation of cellular contents which could precipitate at such low levels. The precipitate then would have balled with the aggregated erythrocytes in the same manner as do precipitated plasma proteins when they occur in the presence of agglutination.

In summary then, these studies seem to indicate that erythrocytes are aggregated by electrolytes as electronegative colloids at pH concentrations below the isoelectric point of hemoglobin and by direct union with metallic salts under the same conditions that function for proteins in general. Gelatin prevents the colloidal aggregation, or agglutination, but not that due to direct chemical union with its accompanying precipitation and coagulation of the erythrocytic contents. The plasma proteins are precipitated at their specific isoelectric points. This precipitation alone is insufficient to cause clumping in the pipettes. If the cells aggregate, on the other hand, because of their own forces and irrespective of the events occurring in the proteins in which they are suspended, then the precipitated proteins serve to qualify and further facilitate clumping by becoming balled with the cells and in turn entangling more cells. Balling and clumping are exaggerated and emphasized with the gentle and regular motion of the rotor, but are inherent in the reactions of blood with bichloride. When the cells are protected from aggregation the rotor is unable to produce clumping. As Ch'u and Forkner[4] found with hand shaking, these same features are naturally present in other methods of shaking. It is merely that most methods of shaking offer enough violence and shift of direction that the colloidal aggregates are prevented from holding, and in that case the precipitated proteins do not find a nucleus for collection and a reciprocal entangling of more cells. Clumping therefore is not in evidence with such methods of shaking, but the underlying potentialities are present nonetheless, and the dangers of clumping are always present. Ch'u and Forkner[4] found this to be particularly true in certain pathologic conditions.

The experimental findings are significant from the standpoint of various clinical and biologic studies other than blood counts, and a brief survey of certain of these seems indicated.

The fact that modifications in pH in the presence of tissue fixatives can effect such marked changes in erythrocytes as lysis or agglutination, respectively, emphasizes anew the importance which Petrunkevitch[6] ascribes to pH in the fixation and staining of tissues. The misconceptions that may occur because of failure to evaluate this factor are discussed in many texts on histologic technique.[7] The fact that differential precipitation of the different elements of the blood exposed to the same fixative may occur simply under the influence of different hydrogen-ion concentrations indicates possibilities for differential analysis of the finer cytology of tissues. Obviously the differences in the way

tissues react to the same stain are due not only to the simple acid-base relationships upon the stains or between the stains and the tissues, but also are determined by the physical state of each element in the tissues, that, in turn, having been determined by the pH of the fixative in relation to the elements in question. These possibilities are very beautifully illustrated in the differential staining of anatomic cross sections which Tobin[8] has obtained with aniline blue used at different hydrogen-ion concentrations.

The lysis of erythrocytes that has been shown to occur in weak solutions of bichloride and of the other fixatives employed is probably due either to solution of cell membranes or to some sort of union of the membranes with the fixatives involved in such a manner as to weaken the membranes and allow extrusion of the contents before coagulation or fixation of the latter can occur. In the case of the weak solutions of bichloride the union is probably with the lecithin of the capsule while the concentration of bichloride, or the pH, or both are insufficient to permit coagulation of the proteins. In stronger concentration the amount of bichloride is sufficient to coagulate or fix the proteins of the cells even as it unites with the lecithin of the capsule. The action of gelatin as a protective colloid applied to the surface of the erythrocytes probably explains its effect in preventing lysis, just as it prevents aggregation.

The capacities of dilute solutions of fixing agents to cause lysis, and especially of very dilute solutions containing mercury at hydrogen-ion concentrations equal to those which obtain in blood and tissues, place significance upon the possibility of equivalent action in local or generalized areas subjected to therapeutic agents of similar nature.

It is significant that the hydrogen-ion concentration of the blood is such as to favor precipitation of plasma proteins and agglutination of erythrocytes in the presence of electropositive metals and colloids, especially those represented by a heavy metal like mercury. The importance of this fact in therapeutic and diagnostic procedures employing such solutions is obvious. When such solutions are used for parenteral therapy, for instance, the events when they meet body fluids must often mark the difference between smooth and stormy reactions. And in such diagnostic procedures as the use of Hayem's solution[9, 10] or other solutions containing bichloride[11-13] in studies of liver function, the ambiguities might well be lessened by regulation of the pH. The recent work of Kunkel[14] with solutions of copper or zinc for a similar purpose certainly shows the importance of pH in the results and interpretations of such tests.

The dangers of unpredicted agglutinations are lessened in the presence of the correct protective colloids for the situation at hand, but the protection from such colloids may be negated by elevation or deprssion of the pH of the environment to that at which the colloid itself is precipitated by the reagent.

The fact that the gentle rotation of blood in the fixed planes set by the Bryan-Garrey rotor brings out the inherent tendency for precipitation and clumping in red blood counts suggests that similar motion might prove useful in biologic and diagnostic procedures that require detection of the phenomena of precipitation or agglutination. In certain respects the method achieves the

same results as are sought at times by vibratory mechanisms.[15] In the present studies the method actually allowed detection of the phenomenon of aggregation when it was masked by the more violent vibration of a shaker or even by hand shaking.

The risks inherent in the use of soft glass containers are once again illustrated in the present studies. Storage of Hayem's or Jörgensen's solution in soft glass containers quickly resulted in massive clumping with whole blood under conditions in which this phenomenon did not occur if the diluents were stored in Pyrex containers. These effects were caused not only by changes in pH due to reaction with soft glass, but also by changes in the content of electrolytes.[5] Since the findings and the interpretation of them are obviously tremendously influenced by the pH and the electrolytes of the test solutions in diagnostic procedures based on precipitative or agglutinative phenomena by means of solutions containing heavy metals, the dangers of change in these factors by improper storage are very real. The reactions between the metal and the biologic substance in question may be encouraged, suppressed, or confused by reactions of other substances, depending upon these factors.

The power of erythrocytes to attract and carry colloidal substances, while recognized, is given far too little consideration in biologic studies. The action of gelatin, and to a lesser extent, that of the plasma proteins in the prevention of aggregation of erythrocytes in these studies illustrates the surface force of the erythrocytes, even as it indicates the colloidal activity of these proteins. The studies of Bloor[16, 17] indicate that after fats have been absorbed from the gut they first are attracted from the plasma to the cells of the blood before chemical changes can take place within the cells. Pennell[18] recognizes surface attraction between erythrocytes and platelets in his concept of a causal relationship between erythrocytes and hemophilia.

CONCLUSIONS

The factors involved in the clumping of blood in red counts made by the aid of mechanical rotation are inherent in the interactions of Hayem's solution with plasma and cells and are merely emphasized by rotation. The capacity of rotors to demonstrate the phenomena of precipitation and aggregation and/or agglutination in blood counts was found useful in elucidation of the factors involved in these phenomena.

The clumping is due to the reactions of mercuric bichloride with the cells and the plasma as a tissue fixative. These reactions are regulated by (*1*) the concentrations of bichloride, (*2*) the relationship of the pH of the diluent to the isoelectric points of the erythrocytic contents and of the plasma proteins, and (*3*) the colloidal activities of intact erythrocytes.

When dilute enough, bichloride may cause lysis. When concentrated enough it causes colloidal aggregation and/or agglutination of the erythrocytes and precipitation of proteins, depending upon the pH. Addition of gelatin to the diluent protects the cells from lysis and aggregation at hydrogen-ion concentrations below pH 7.0, but does not affect the precipitation of proteins.

Aggregation of the erythrocytes and precipitation of the proteins under the action of bichloride occur independently of each other. Aggregation alone may simulate clumping. Precipitation of the plasma proteins alone does not cause clumping. When aggregation accompanies precipitation, characteristic clumping occurs.

The findings are disussed from the standpoint of their significance in relation to problems other than blood counts. They bear upon the phenomena of lysis, precipitation, and agglutination, upon diagnostic and therapeutic measures which involve colloidal or precipitative reactions, and upon the differential responses of tissues to fixation and staining.

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MEASUREMENT OF THE ELECTRIC RESISTANCE OF HUMAN BLOOD; USE IN COAGULATION STUDIES AND CELL VOLUME DETERMINATIONS

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THE measurement of electric resistance and conductivity is a universally recognized physical-chemical procedure which has had wide use in biologic investigations. It has been successfully used in the study of cell permeability, muscle physiology, and so forth, and attempts have been made to measure electric resistance changes during the blood coagulation process. Most of the results obtained have indicated that there is no blood resistance change during coagulation. A review of the literature reveals that most of the work done on this subject was published before 1926 and centers around resistance or conductivity as related to cell volume and coagulation. Stewart and other[1-5] have shown that the resistance of blood is directly proportional to the cell volume. Blood may be thought of as a suspension of cells, which are very poor conductors, and plasma, whose conductivity depends upon the concentration of electrolytes, particularly sodium chloride.[6] Sigman and co-workers[7] measured the resistance of beef blood flowing through a tube. They reported that the resistance decreases with increasing flow velocity. Other workers[8-10] have employed resistance measurements in studies of hemolytic and osmotic behavior of the red cell. Resistance of packed red cells, normally thirty to forty times the resistance of plasma, is reduced to a value comparable to that of plasma by saponin hemolysis.

Most authors were unable to demonstrate reproducible electric resistance changes during the complex process of blood clotting.[11-14] In the only recent report, Graff and co-workers[15] say that there are irregular resistance changes during clotting, not related to clotting time, and that the resistance of heparinized blood measured in vitro steadily increases in the first half-hour after obtaining the blood sample.

In the present paper a reliable and practical experimental method is described for the measurement of electric resistance of blood and other fluids. Observations are presented to show that this method enables one to make reproducible resistance versus time measurements on whole blood and that the varying observations of previous investigators may have been due to inadequate technique. These observations apply to changes in blood resistance during coagulation and to the relation between blood resistance and cell volume fraction.

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METHOD

The application of the classic method of Kohlrausch and Holborn[16] and its elaboration worked out by Washburn and others has been widely accepted for the measurement of the electric resistance of colloid systems and suspensions. Taylor and Acree,[17] Washburn and Weiland,[18, 19] and others[20-25] have given excellent discussions of the theoretic principles. It is beyond the scope of this paper to discuss technical details other than those applying especially to the determination of the resistance of blood. Most of the experimental arrangements for blood resistance measurements reported in the literature appear to have been unduly complicated and time consuming and intended for a limited number of measurements. We believe we have designed a setup which can be used to study the clotting of blood, permitting rapid successive resistance measurements. The method has high reliability of single measurements, and it could be adapted to measurement of other biologic materials.

Fig. 1 shows the measuring tube and electrodes. The tube, made of Pyrex, was 12 mm. in diameter and could be held in any test tube rack. The electrodes, consisting of lightly platinized* platinum plates 0.01 inch in thickness, ¼ sq. cm. in area, and ½ cm. apart, were designed to be large enough to avoid the errors arising in the use of wire point and small electrodes and yet to require only about 1.5 c.c. of blood or other fluid. Platinized electrodes eliminated the necessity of extrapolating the measurements to infinite frequency as required in the case of unplatinized (bright) electrodes, and they gave a sharper end point of impedance balance. Also, measurements during clotting made with unplatinized electrodes often showed discrepancies which could be explained by imperfect adherence of the clot to the electrodes. The electrodes were cleaned satisfactorily by connecting them as cathode in 20 per cent sodium hydroxide and applying 3 volts for two minutes. The adherent fibrinous material loosened by this process readily washed off with hot distilled water.

The position of the electrodes in relation to the measuring tube and blood clot determines the result of measurement to some extent. In the geometry used in the present experiments the electrodes were entirely contained within the clot, and they remained in the clot during retraction (see Fig. 2). This enabled us to study the change of resistance during clot retraction for a considerable length of time. Unfortunately, some previous investigators did not describe their electrode arrangements. It was found by us that if electrodes are placed too distantly or in an inconvenient way the clot may break off during the process, allowing plasma to interfere with the clot resistance measurement. The results obtained with such latter electrode arrangements are irreproducible.

An audio frequency oscillator† capable of producing an alternating current frequency from 20 to 200,000 cycles per second was used to generate power. Since measurements with the platinized electrodes showed negligible variation with frequency, 1,000 cycles A. C. was used through the experiments.

The impedance bridge‡ provided an accuracy adequate for our purposes with a probable error of less than 1 per cent and a maximum error of 2 per cent. The amplitude of the applied voltage was 0.25 volts root mean square. Capacitance of the measuring cell circuit was negligible and compensation did not alter the readings. Our parallel control observations showed that the behavior of clotting blood was not changed beyond the range of experimental error by the current used in the measurements.

Instead of using the conventional telephone bridge balance indicator, an oscilloscope§ was used. The horizontal sweep was connected to a 60-cycle saw-tooth power supply. The

*Electrodes were platinized with Lummer-Kurlbaum solution: 0.3 Gm. platinum chloride, 0.003 Gm. lead acetate dissolved in 10 c.c. of distilled water. Six volts were applied, each electrode connected as a cathode, for thirty seconds. Electrodes were cleaned by 2N sulfuric acid, connected as anode, 3 volts for 1 and one-half to 2 minutes. Electrodes had to be replatinized when the platinum black surface became insufficient.

†Hewlett-Packard, Palo Alto, Calif., model 200 C.

‡General Radio Co., Cambridge, Mass., type 650-A.

§Cathode-ray-oscillograph, Type 224, Du Mont Laboratories, Passiac, N. J.

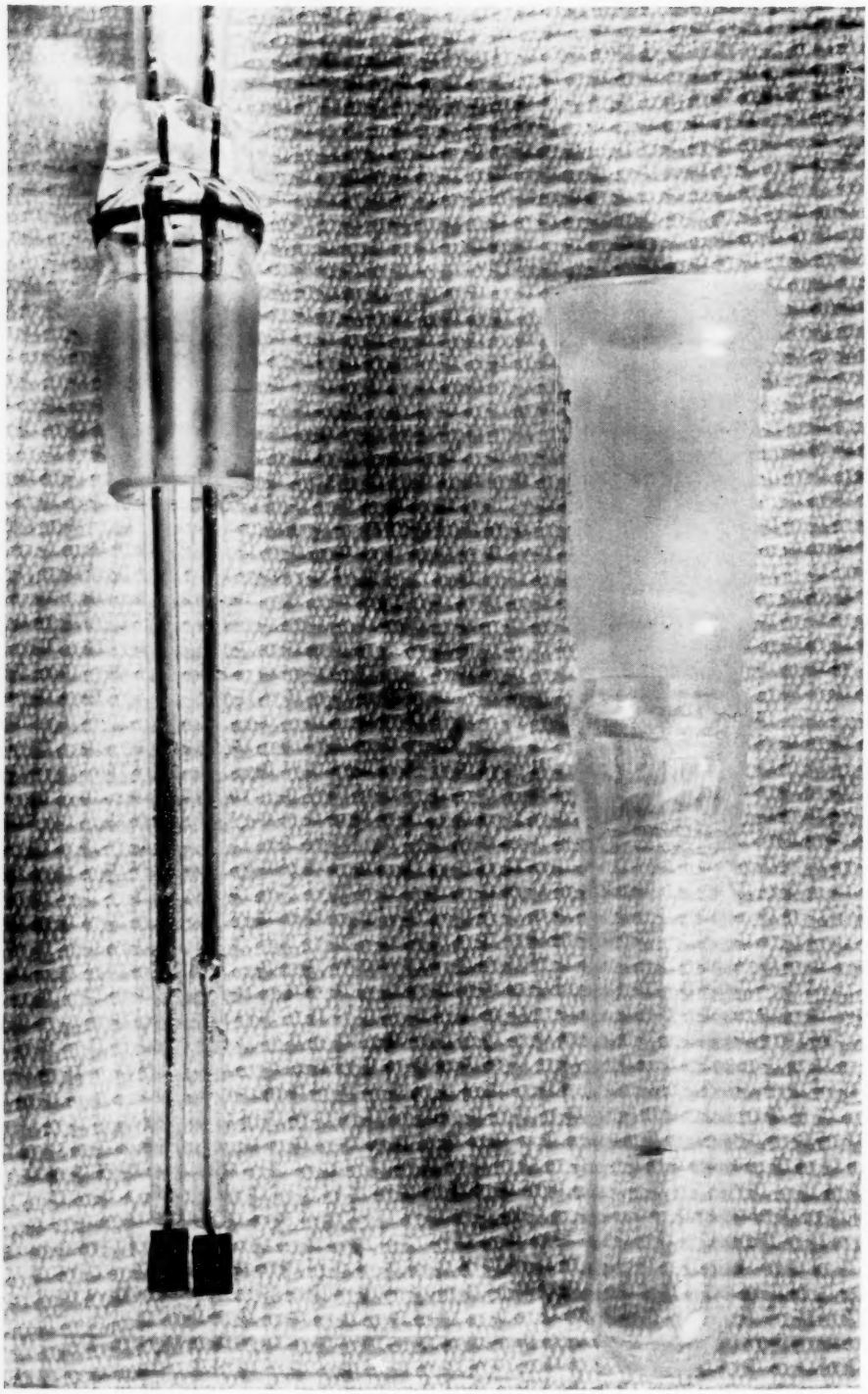

Fig. 1.—Measuring tube and electrodes.

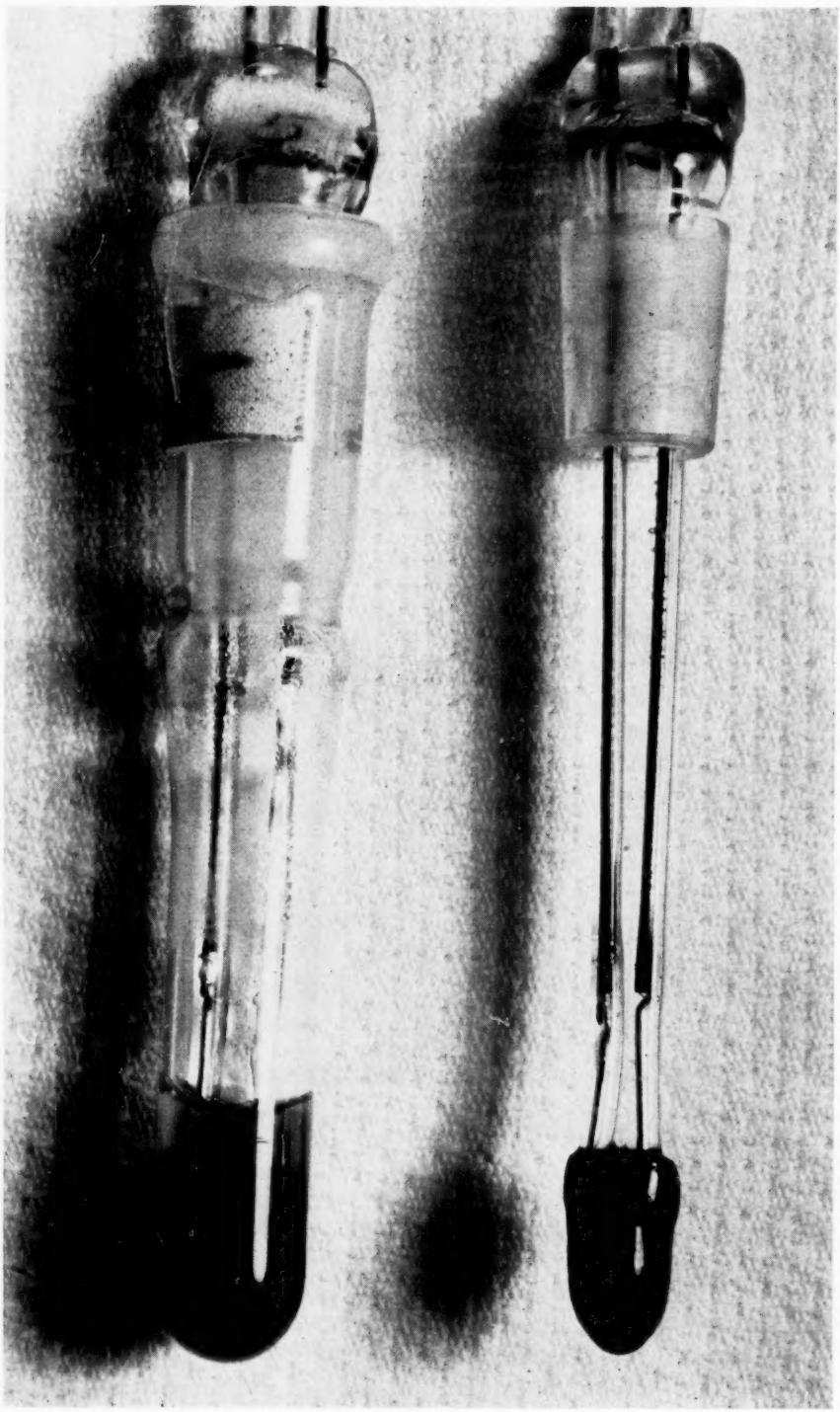

Fig. 2.—Appearance of retracted clot in situ in the measuring tube and its adherence to the electrodes on their removal from the tube.

vertical sweep showd the amplified 1,000 cycle A. C. voltage appearing between the unbalanced junctions of the impedance bridge. At balance, the vertical amplitude reached a well-defined minimum.

By means of a selector switch and parallel circuits, six different samples could be studied at one time. All determinations were made in a constant temperature water bath set at 37° C. (See Fig. 3). An increase in temperature of 1° C. produced an approximate 2.5 per cent decrease in resistance. The conductivity cell constant was measured with 0.1 KCl and all measurements were converted to specific resistance in ohm-centimeter units by the relation:

$$\text{Specific resistance at } 37^\circ \text{ C.} = \frac{\text{Measured resistance of blood}}{0.0158 \times \text{Measured resistance of 0.1N KCl}}$$

where the factor 0.0158 is the specific conductance of 0.1N KCl at 37° C.

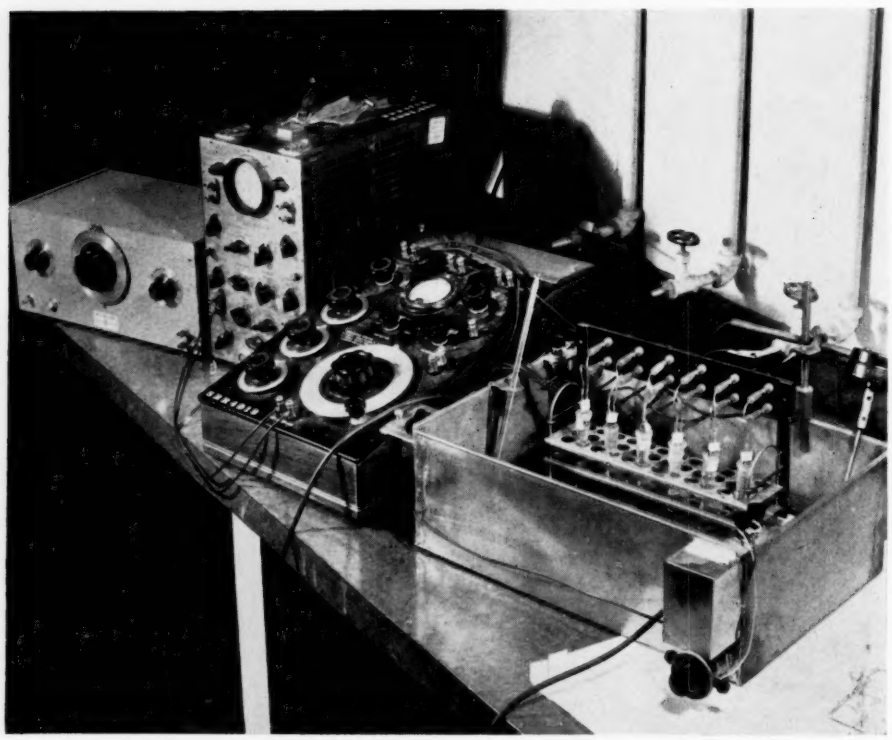

Fig. 3.—Left to right: audio frequency oscillator, oscilloscope, impedence bridge, water bath and stand with six measuring tubes connected for making measurements.

OBSERVATIONS

Resistance Changes During Blood Coagulation.—1.5 c.c. of blood drawn with a dry syringe were carefully placed in the measuring tube; the electrodes were inserted, and measurements were made at frequent intervals for forty minutes. Parallel clotting time determinations were performed by a modified Lee-White method (½ c.c. of blood; two tubes 10 mm. in diameter). The results are shown in the two curves in Fig. 4, with time plotted against specific resistance in ohm-centimeter units. The lower curve represents the

average of measurements on sixteen blood normal specimens, while the upper one is composed of the average of fourteen polycythemic blood samples. All blood samples showed clot retraction. The curves for two individuals together with the clotting times are shown in Fig. 5. For the first few minutes the resistance value was constant at a level indicative of the cell volume per cent. Then, at the time clotting occurred, the resistance started to increase. This increase, which is related to clot retraction, has been found to continue for at least seven or eight hours. Each of the determinations followed this pattern, with variations occurring in the magnitude of resistance, point of beginning resistance increase, and slope of the curve. The higher value for the one-minute determination could be explained by the temperature adjustment and possibly by insufficient time for thorough wetting of the electrodes. The slight drop in temperature of the blood while in the syringe would increase the resistance, 2.5 per cent for each degree centrigrade.

Definite substantiation of clotting as the factor responsible for the time-resistance curve pattern described was obtained by observations on blood rendered incoagulable by the in vitro addition of heparin (0.1 mg.) or oxalate (1.8 mg. ammonium and 1.2 mg. potassium oxalate). The unclotted blood gave no significant change in resistance over a one-hour period as seen in Fig. 6 which also shows the same blood, without any addition of an anticoagulant, giving the typical clotting pattern. Very slight variations in resistance were the probable effect of red cell sedimentation to produce changing cell

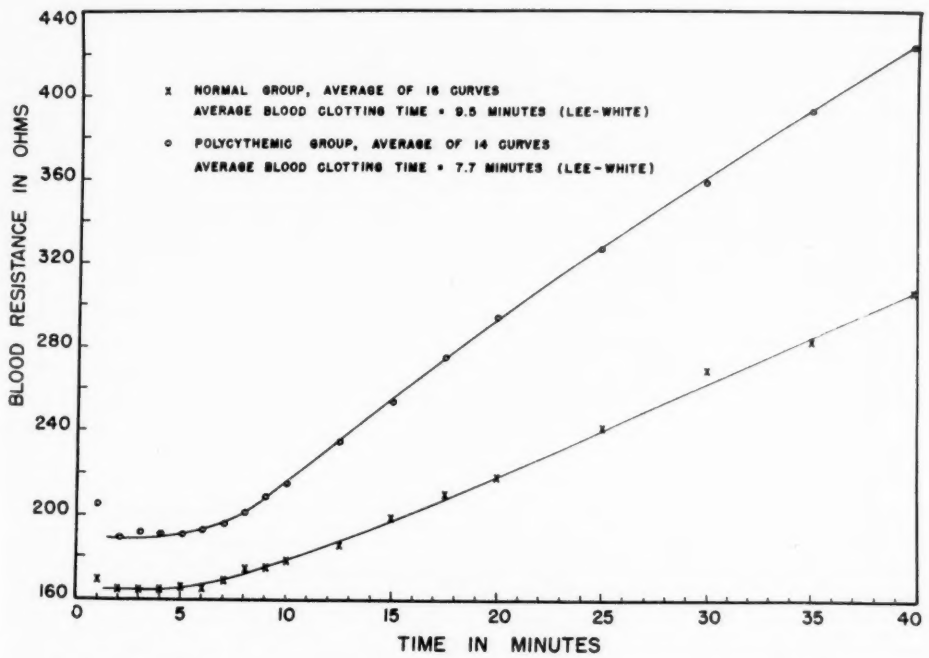

Fig. 4.—Curves of the average blood resistance during clotting for sixteen normal and fourteen polycythemic subjects.

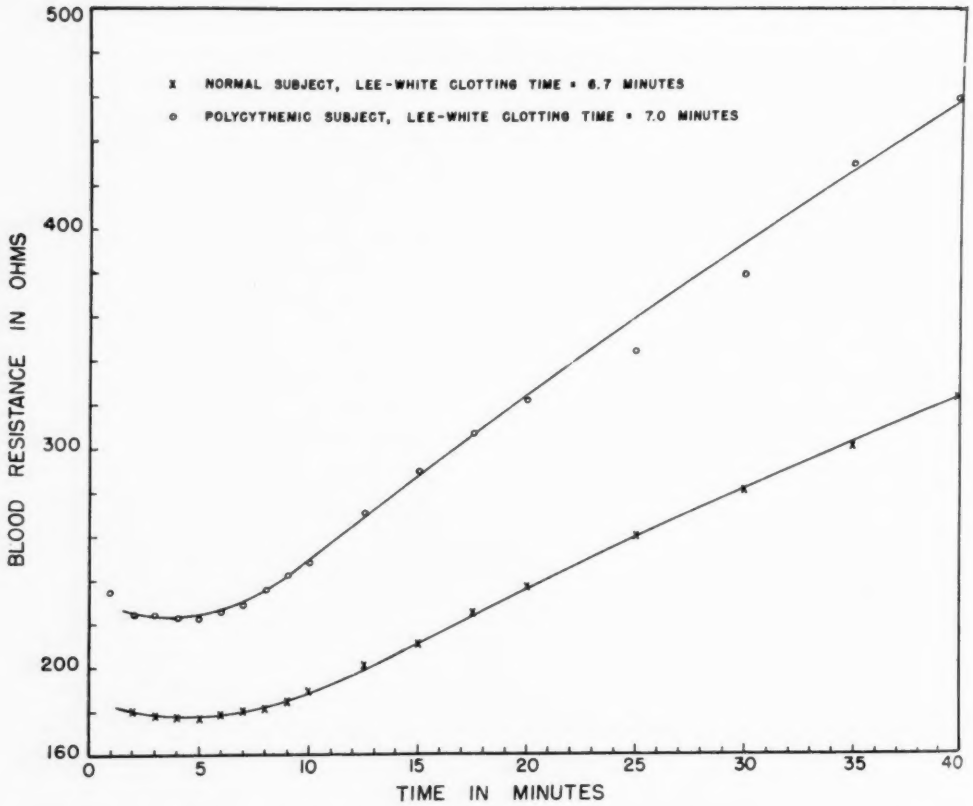

Fig. 5.—Blood resistance curves during clotting for a normal subject and a polycythemic subject.

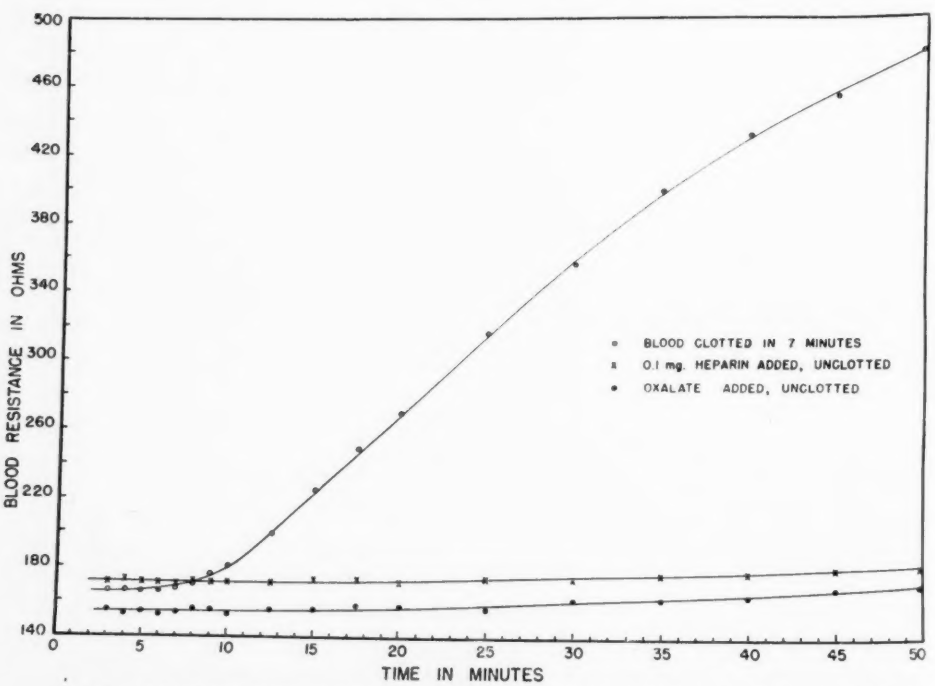

Fig. 6.—Resistance measurements showing the effect of rendering blood incoagulable by the addition of anticoagulants such as heparin and oxalate. The changes during clotting for the blood of the same subject are shown for comparison.

concentrations around the electrodes. It was found that shaking or stirring the unclotted blood increased the resistance, which dropped in one or two minutes to a constant value about 6 per cent lower as the cells settled.

*Resistance and the Cell Volume Fraction of Blood**.—The electric resistance of plasma is much lower than that of blood cells alone. Whole blood resistance is determined by the intrinsic resistance of plasma and cells and by the relative amount of cells and plasma present in the blood. It also has been shown that the shape of the red blood cells influences whole blood resistance. Ponder[4, 5] has presented a formula for calculating the cell volume fraction of a sample of blood from the measurement of the resistance of both the unclotted blood and its plasma separated by centrifugation.

$$\text{Cell volume fraction} = \frac{\phi - 1}{\phi + \frac{1}{X}}$$

Where $\phi = \frac{\text{resistance of blood}}{\text{resistance of plasma}}$ and X is the form factor dependent on the shape of the cells, assumed to equal 1.10 for red cells in plasma. Also, cell volume per cent = cell volume fraction × 100.

In the present study the cell volume per cent was determined by both electrical resistance measurements and centrifugation (Wintrobe tube; heparinized blood; 2,500 revolutions per minute for one-half hour; reading represent red cell volume) on aliquot samples of venous blood. Plasma values averaged 63.2 ohm-centimeters with a range of 61.0 to 66.8 ohm-centimeters. The blood resistance, taken as the average value of the three-, four-, and five-minute readings of the time-resistance curve, ranged from 131.2 to 230.9 ohm-centimeters. A comparison of the results obtained by the two methods revealed that the resistance determinations were 7.7 per cent lower than the centrifuge values in normal subjects and 7.8 per cent lower in patients with polycythemia vera for average values (see Table I). In three leucemia blood specimens with an average white count of 96,200 the resistance method compared 5.7 per cent higher than the centrifuge method. This may be explained by the fact that while only the red cell volume was recorded for the centrifuge cell volume, the resistance measurement accounted for the high, poorly conducting white cell volume as well as the red cell volume. For normal or only slightly elevated white counts, the white cell resistance factor was very small.

The cell volume per cent can readily be determined from resistance measurements by taking the ratio of blood to plasma resistance (ϕ) and re-

*Since the precise meanings of the terms cell volume and hematocrit are at the present time confused, we deem it advisable to clarify certain terms used in this paper. Hematocrit is used in its strict sense to refer to the volume of packed red cells, expressed as a per cent, obtained by centrifugation of 1 c.c. of blood in a tube 100 mm. long. The term cell volume has been used to designate the proportion of cells by volume in a given sample of blood, the total cell volume of an animal, or volume of an individual cell. In this paper the term cell volume fraction is used to indicate the proportion of cells by volume in venous blood irrespective of the method used in its determination. The volume per cent of cells in the blood sample is termed cell volume per cent irrespective of its method of determination. Since the volume of white cells, unless abnormally increased as in leucemia, is very small, the cell volume per cent approximates the red cell volume.

ferring to a curve of the cell volume per cent values calculated from Ponder's equation plotted against ϕ as in Fig. 7. The values obtained by the centrifuge method are plotted against ϕ for comparison. Table II lists some pertinent data on subjects used in these measurements.

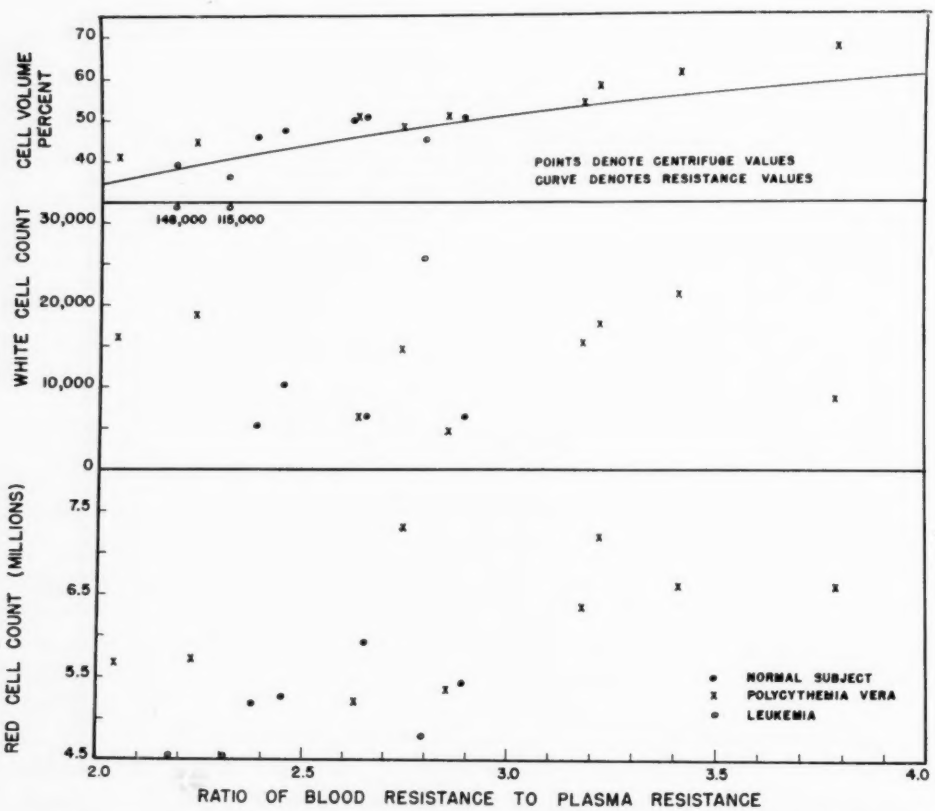

Fig. 7.—Ratio of blood to plasma resistance (ϕ) plotted against cell volume per cent, white count, and red count for seventeen subjects. The absence of a simple relation between blood resistance and white and red counts is shown. There is a direct relation of resistance to cell volume (chiefly red cell). The curve represents cell volume per cent values obtained by calculation from Ponder's formula using the ratio of blood resistance to plasma resistance (ϕ).

In order to obtain a wider range, heparinized blood was diluted and concentrated by varying the proportion of cells to plasma, and cell volumes were determined by the two methods on aliquot samples. The blood resistance measurements were obtained immediately after shaking, before settling had occurred. Results, shown in Table III, indicated that the resistance determinations were 6.1 per cent lower for the normal group, 8.1 per cent lower for polycythemic groups, and 2.0 per cent lower for the leucemic group. The combined normal and polycythemic groups gave a value of 7.3 per cent.

The level of blood resistance showed no direct relation to hemoglobin, red cell count, white cell count, or platelet count. Fig. 7 shows the red and white counts plotted against the blood-plasma resistance ratio (ϕ).

TABLE I. CELL VOLUME PER CENT OF VENOUS BLOOD DETERMINED BY ELECTRIC RESISTANCE AND CENTRIFUGE METHODS

GROUP	NUMBER OF SAMPLES	AVERAGE CELL VOLUME PER CENT		CELL VOLUME PER CENT DIFFERENCE*	AVERAGE RED COUNT	AVERAGE WHITE COUNT
		RESISTANCE	CENTRIFUGE			
Normal	5	45.5	49.2	−7.7	5,490,000	7,100
Polycythemic	9	48.8	52.9	−7.8	6,230,000	13,630
Leucemic	3	42.4	40.1	+5.7	4,570,000	96,200

*Cell volume per cent difference represents the difference between the resistance and centrifuge cell volumes expressed as per cent of centrifuge value.

TABLE II. BLOOD AND PLASMA RESISTANCE DATA FOR NORMAL, POLYCYTHEMIC, AND LEUCEMIC SUBJECTS

SUBJECT	SPECIFIC RESISTANCE (OHM-CM.)		RES. BLOOD / RES. PLASMA	CELL VOLUME PER CENT		RED CELL COUNT	HEMOGLOBIN	WHITE CELL COUNT	PLATELETS
	BLOOD	PLASMA	ϕ	RESISTANCE	CENTRIFUGE				
				Normal					
J. M.	147.6	62.1	2.38	41.8	46	5.24	13.0	5,500	—
A. C.	163.8	66.8	2.45	43.2	48	5.29	14.5	10,100	320,000
L. D.	160.7	61.0	2.63	46.0	50	—	—	—	—
B. W.	173.6	65.6	2.65	46.3	51	5.94	14.0	6,300	—
L. D.	176.3	61.0	2.89	49.7	51	5.48	13.0	6,600	240,000
				Polycythemia					
A. F.	131.2	64.3	2.04	35.2	41	5.68	11.0	15,750	730,000
A. F.	143.5	64.4	2.23	39.0	45	5.72	10.6	18,000	680,000
W. M.	169.1	64.4	2.63	46.0	51	5.20	15.0	6,100	400,000
W. P.	180.0	65.6	2.74	47.7	48	7.30	12.0	14,500	340,000
A. I.	174.7	61.3	2.85	49.2	51	5.36	13.5	4,700	290,000
M. P.	205.1	64.5	3.18	53.4	54	6.35	14.8	15,400	400,000
A. P.	199.8	62.0	3.22	53.8	58	7.20	15.5	17,500	350,000
A. P.	223.5	65.5	3.41	55.8	61	6.70	15.6	21,200	350,000
M. R.	230.9	61.0	3.78	59.2	67	6.59	15.0	8,750	370,000
				Chronic Lymphatic Leucemia					
M. R.	137.8	63.2	2.18	38.2	39	4.45	11.6	148,000	210,000
M. R.	143.0	62.0	2.31	40.6	36	4.46	11.5	115,000	250,000
P. B.	170.3	61.0	2.79	48.3	45.3	4.81	12.0	25,700	170,000

TABLE III. CELL VOLUME PER CENT OF VARIOUS DILUTIONS AND CONCENTRATIONS OF HEPARINIZED VENOUS BLOOD

SUBJECT	NUMBER OF SUBJECTS	NUMBER OF OBSERVATIONS	AVERAGE CELL VOLUME PER CENT		CELL VOLUME PER CENT DIFFERANCE*
			RESISTANCE	CENTRIFUGE	
Normal	5	10	53.5	55.0	−6.1
Polycythemic	9	16	50.9	55.4	−8.1
Leucemic	3	4	33.9	34.6	−2.0
Normal and Polycythemic	14	26	51.9	56.0	−7.3

*See footnote to Table I.

DISCUSSION

Our results show blood resistance measurement to be of value in the study of blood coagulation, both as a method for the detection of the clotting time and as a means for obtaining the quantitative measurement of the rate of clot retraction. The advantage in the use of electric resistance lies in the fact that a dynamic process such as blood coagulation may be studied under controlled conditions without disturbing the process by the making of measurements.

Emphasis must be placed upon the importance of the geometric orientation of the electrodes to the clotting blood and retracting clot in the evaluation of observations and data. We located our electrodes at the central part of the blood and within the retracting clot. As soon as the blood has clotted, clot retraction begins by the contraction of the fibrin network which pulls the large elements or cells together into a dense mass, thus displacing the serum to the periphery. This process produces increases in resistance measurements because it simultaneously increases the concentration of poorly conducting cells and decreases the concentration of serum, a good conductor, between and around the electrodes. On this basis it is possible to relate our observed changes in resistance during clotting, as graphically demonstrated by the time-resistance curve, to blood coagulation events. Prior to clot formation there is no significant change in resistance. The clotting time and start of clot retraction are marked by the first increase in resistance. Thus the clotting time may be determined with the elimination of motion, the source of considerable variation and inconsistency in most methods in common use.

The subsequent increases in resistance result from retraction of the clot. Therefore the slope of the rising portion of the time-resistance curve may be assumed to correspond to the rate of clot retraction and to serve as a quantitative measure of this process. Since there has been no method previously available for a comparable quantitative study of clot retraction, such measurements may serve to detect significant differences in and variations of this process in disease. The method also may provide a means to determine the effect of chemical and therapeutic agents upon clot retraction.

Our results differ from those reported by Graff and co-workers[15] who obtained an increase in resistance of normal blood during the first ten minutes, unrelated to clotting time, while heparinized blood gave a rapid progressive increase in resistance for twenty to thirty minutes. The description of the method given by these authors is not complete enough to allow us to compare their data with ours. Certain important features of their method such as the use of 60 cycles A.C. and applied voltage of 5 volts root mean square appear to be at variance with accepted procedures which have been thoroughly worked out for conductivity measurements.

It is of investigative interest to compare the cell volume fraction calculated from resistance measurements with the centrifuge hematocrit. It has been known for some time[3] that hematocrit values obtained by separation of cells from plasma in the centrifuge do not give the true cell volume fraction. This

would be true only if the centrifuge would pack the cells so closely that all intercellular space and plasma would be excluded from among the cells. For example, Miller[26] and Kennedy and Millikan[27] demonstrated that in order to achieve such perfect separation, centrifugal force of a magnitude that would destroy the cells themselves would be required. There are a number of papers available which compare various methods for the determination of the cell volume fraction with the centrifuge method. Kennedy and Millikan[27] and Shohl and Hunter[28] reported values obtained by using dye dilution of plasma 10 to 12 and 4.5 per cent, respectively, below the centrifuge hematocrit. Chapin and Ross[29] found the cell volume fraction determined by dye dilution (T-1824), protein dilution, and the use of red cells tagged with radioactive iron to be an average of 8.5 per cent lower than the cell volume fraction obtained by centrifugation. Ponder and Saslow[30] calculated the cell volume fraction in whole blood from direct measurement of the dimensions of the individual red cell and their number. This calculated value was in close agreement with the dye dilution determination of the cell volume fraction of the same blood. Stewart[3] used the method of conductivity and also obtained values below the centrifuge hematocrit (5 to 6 per cent). The results presented in this paper indicate that the cell volume fraction determined by electric resistance measurements is 7.7 per cent lower than values obtained by centrifugation. Thus the dye dilution, protein dilution, tagged red cell, direct measurement, and electric resistance methods agree very well in the determination of the fractional volume of red cells in blood, while the value obtained in the centrifuge hematocrit is consistently high because of plasma trapped between the packed red cells.

SUMMARY

A reliable rapid method for the measurement of electric resistance of small amounts of blood or similar material is presented.

A pattern of change in the resistance of blood during coagulation is described. These resistance changes make possible the determination of the clotting time with elimination of inconsistencies caused by motion and offer a quantitative means for the study of clot retraction. In view of the fact that no method has been available previously for a comparable quantitative study of clot retraction, electric resistance measurements may serve to detect significant variations of this process in disease beyond our present knowledge.

By means of the ratio of blood resistance to plasma resistance, the cell volume fraction of a sample of blood may be calculated. Cell volumes determined by resistance measurements were found to average 7.7 per cent lower than the hematocrit as determined by centrifugation.

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THE EFFECT OF CHOLINE, METHIONINE, AND LOW FAT DIET ON THE LIFE EXPECTANCY OF PATIENTS WITH CIRRHOSIS OF THE LIVER

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IN A previous communication from this clinic a regimen for the treatment of cirrhosis of the liver was described.[6] This regimen was based upon two assumptions: first, that fatty changes in the liver ordinarily precede the more characteristic anatomic lesions of cirrhosis; second, that fatty changes in the liver may be dietary in origin, especially in the alcoholic patient, and hence might be comparable to the changes observed in experimental animals placed on deficient diets. The clinical course of several patients was described. The results attributed to the therapy were striking in each instance. It was pointed out, however, that not all patients had responded so dramatically and that spontaneous remissions have long been known to occur in the absence of any specific therapy. Interpretation of the efficacy of the therapeutic regimen therefore had to await the study of an increased number of patients over a longer period of time.

METHOD OF STUDY

The present report summarizes the clinical experiences of the past five years, during which time some 224 patients with cirrhosis of the liver were studied. The fate of these patients is compared with that of a similar control group of patients treated in the same hospital under similar circumstances prior to the use of the present therapeutic regimen.

The therapeutic regimen used in the treatment of the present series of patients is summarized in Table I.

Diet.—The protein intake was maintained as near 100 Gm. per day as possible so that presumably adequate supplies of methionine and cystine as well as other essential amino acids were provided. The fat intake was limited to approximately 50 Gm. per day. It seemed unreasonable to burden further a fatty liver with additional supplies of fat, since it was assumed and later demonstrated experimentally[4] that under some circumstances at least much of the liver fat was exogenous in origin. Animal fat was particularly avoided since cholesterol is known to be one of the most potent stimulants to fat infiltration.[7] Such fat infiltration is reported by some observers to be exceedingly resistant to lipotropic agents.[2] Carbohydrate was given ad libitum (400 to 600 Gm.) and in sufficient quantities to spare dietary protein for more essential purposes. The total caloric value of the diet was approximately 3,000 calories.

Drugs.—More specific attempts were made to supply choline and methionine. At first, 1 Gm. of choline chloride was given daily in divided doses. Since it

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TABLE I. THERAPEUTIC REGIMEN

Diet	
Protein	90-100 Gm. +
Fat (chiefly vegetable)	50 Gm.
Carbohydrate	Adequate to supply caloric needs
Drugs	
Choline hydrochloride	1-8 Gm.
Methionine (1 qt. skimmed milk)	0.9 Gm. +
Vitamins	
A	10,000 U.S.P. units
D	800 U.S.P. units
Abstinence from alcohol	

had been emphasized by some observers[3, 5] that large doses of choline given over a prolonged period of time were required once liver damage was established, the dosage was increased cautiously on the basis of the patient's tolerance. Few were able to take more than 6 Gm., and none more than 8 Gm. without experiencing gastrointestinal symptoms which were thought to be referable to the choline chloride. Most patients took and tolerated 3 to 5 Gm. daily very well.

Since pure methionine was obtainable with difficulty and at a considerable cost at the outset of these observations, milk was used as a source thereof. All patients consumed at least one quart of milk (skimmed to avoid unnecessary animal fat) daily. This is equivalent to approximately 0.9 Gm. of methionine.

Vitamins.—Supplements of the fat-soluble vitamins A (10,000 U.S.P. units) and D (800 U.S.P. units) were given in order to compensate for the restrictions of dietary fat. Supplements of the B complex were not used so as not to complicate further the evaluation of the therapeutic program.

Alcohol.—Alcohol consumption was prohibited, not because of any fear of the alcohol per se, but rather because the therapeutic regimen usually was followed inadequately if the patient continued to imbibe. Whenever it was apparent that cooperation in this respect was not probable, the patient was kept in the hospital for long periods of time (six to eight months) and often on repeated occasions. No similar effort was made to govern the alcohol intake of the control group. While it is difficult to evaluate the relative extent to which the members of the two groups heeded the warning to abstain, it is very probable that far better control was maintained in the experimental group.

CASE MATERIAL

Those patients admitted to the St. Louis City Hospital during the eleven-year period beginning April 1, 1935, and upon whom the diagnosis of cirrhosis of the liver was made are the basis of the present report. The patients were divided into two groups: the experimental and control groups. The former included those patients with cirrhosis who were admitted to the hospital after April, 1942, and who were treated according to the therapeutic regimen described. The control group consisted of those patients who were admitted prior to April 1, 1942, and who were not treated according to any particular therapeutic regimen. For the most part these patients received a high carbohydrate diet. An occasional omentopexy was done.

Of the entire series of 647 patients, 112 were omitted from consideration in this study because of doubts concerning the validity of the diagnosis. There remained 224 patients in the experimental series and 311 patients in the control group. Autopsy or biopsy material was available on approximately 65 per cent of these patients. The remainder of the diagnoses were based upon unmistakable clinical and laboratory data. (See Table II.)

TABLE II. CASE MATERIALS

	EXPERIMENTAL (4/1/42 TO 1/1/46)	CONTROL (4/1/35 TO 4/1/42)	BOTH (4/1/35 TO 4/1/46)
Total number of hospital admissions	56,217	63,812	120,029
Diagnosis of cirrhosis	263	384	647
Diagnosis doubtful	39	73	112
Total number of patients with cirrhosis	224	311	535
Incidence (per cent of total admissions)	0.40	0.49	0.45

Although the number of patients in each of the two series was thought to be sufficiently large to insure their comparability, an effort was made to compare the two series in as many respects as possible.

The incidence of the disease among the total hospital admissions, the sex incidence of the disease, the age of the patients, their occupations, the presenting complaints, the initial physical findings, and the laboratory data were approximately the same in the two series of patients. Because of the rationale upon which the therapeutic regimen is based, the dietary and alcoholic histories as well as the size of the livers deserve special consideration.

Alcohol Consumption.—Objective data regarding alcohol consumption before admission are difficult to secure. Both the patient and the physician are likely to color the information as the result of their own past experiences. The following arbitrary classification of alcohol consumption is the basis for the present evaluation:

One plus, occasional consumption of alcoholic beverages but not to the point of intoxication; two plus, average daily consumption of one or two highballs or two to four glasses of beer; three plus, average daily consumption of a pint of hard liquor or four quarts of beer; four plus, all others.

TABLE III. HISTORY OF ALCOHOLISM IN PATIENTS WITH CIRRHOSIS

	EXPERIMENTAL		CONTROL		BOTH	
	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
No data	33	15.5	94	60.1	127	34.4
Data	180	84.5	62	39.9	242	65.6
Total	213	100.0	156	100.0	369	100.0
Alcoholism						
Denied	12	6.7	1	1.6	13	5.4
1+	24	13.3	0	0.0	24	9.9
2+	20	11.1	3	4.8	23	9.5
3+	21	11.7	21	33.9	42	17.3
4+	103	57.2	37	59.7	140	57.9
Total	180	100.0	62	100.0	242	100.0

Most would probably agree that those characterized by three and four plus alcohol consumption should be classified as chronic alcoholics. The data presented in Table III reveal that 70 to 75 per cent of the patients in these two series were chronic alcoholics.

Dietary Histories.—Accurate data on dietary habits are equally difficult to secure and must also necessarily involve subjective factors both on the part of the patient and the interrogator. Nonetheless an attempt was made to evaluate the diets in approximately half the patients. In Table IV it will be observed that 75 per cent or more of the patients were existing on inadequate food intake. Deficiencies in protein, fresh fruits, and vegetables as well as total caloric intake usually were obvious. Such deficiencies were correlated with alcohol consumption. Patients readily admitted that when they were drinking they did not eat.

TABLE IV. DIETARY HISTORY OF PATIENTS WITH CIRRHOSIS

	EXPERIMENTAL		CONTROL		BOTH	
	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
No data	109	51.2	143	91.7	252	68.3
Data	104	48.8	13	8.3	117	31.7
Total	213	100.0	156	100.0	369	100.0
Adequate	16	15.4	0	0.0	16	13.7
Average or fair	8	7.7	0	0.0	8	6.8
Inadequate	80	76.9	13	100.0	93	79.5
Total	104	100.0	13	100.0	117	100.0

The 20 or 25 per cent of the patients whose consumption of alcohol was not considered to be excessive and whose diet was not necessarily inadequate are of considerable interest. While it is undoubtedly true that some of these individuals may have been inaccurate in relating their histories, it is equally certain that lesions of the liver indistinguishable clinically or anatomically from those of typical alcoholic cirrhosis occurred in the absence of gross dietary defects or excessive alcohol consumption. Vague histories of previous jaundice were obtained in some of these patients. Whether or not these were instances of infectious hepatitis cannot be stated with any certainty.

TABLE V. LIVER SIZE

	EXPERIMENTAL (%)	CONTROL (%)	BOTH (%)
Size unknown	16.6	42.9	26.6
Size recorded	83.4	57.1	73.4
Total	100.0	100.0	100.0
No enlargement	23.2	20.1	22.1
1 cm. below costal margin (Rt. M.C.L.*)	3.8	6.8	4.8
2 cm. below costal margin (Rt. M.C.L.)	17.0	16.9	17.0
3 cm. below costal margin (Rt. M.C.L.)	23.7	24.7	24.0
4 cm. below costal margin (Rt. M.C.L.)	24.2	16.9	21.8
5 cm. below costal margin (Rt. M.C.L.)	5.4	11.2	7.4
6 cm. or more below costal margin	2.7	3.4	2.9
Total	100.0	100.0	100.0

*M.C.L., Mid-costal line.

Size of Liver.—Enlargement of the liver was one of the most constant physical findings. More than 75 per cent of the patients had definitely palpable livers. In most instances the enlargement was considerable, as is evident upon inspection of Table V. It is presumed that such enlargement was the result of fat infiltration and hypertrophy of liver cells. Lipotropic substances should be of value under these circumstances.

FINDINGS

Survival.—The fate of the patients in the two groups as of Oct. 1, 1947, is summarized in Table VI. One hundred sixty-nine or 75.5 per cent of the experimental group were known to be dead as compared with 266 or 85.5 per cent of the control group. This comparison permits no conclusion regarding the merits of the therapeutic regimen since the period of observation for the experimental group was several years shorter than that of the control group. The observed periods of survival after the onset of initial symptoms varied from 21.0 to 73.0 months in the experimental group as compared with 78.0 to 176.0 months in the control group. The average period of survival for the period of observation was 40.2 and 122.0 months respectively.

TABLE VI. FATE OF PATIENTS WITH CIRRHOSIS (10/1/47)

	EXPERIMENTAL		CONTROL	
	NUMBER	PER CENT	NUMBER	PER CENT
Dead	169*	75.5	266†	85.5
Living	37	16.5	6	2.0
Fate unknown	18	8.0	39	12.5
Total	224	100.0	311	100.0

*61.3 per cent autopsied.
†62.2 per cent autopsied.

Mortality.—It was not possible to utilize all these cases since the cause of death in some instances was not thought to be directly or indirectly related to the liver disease. In still other instances the lack of adequate data regarding the time of onset of the disease precluded use in this comparison. The experimental series was thus reduced to 139 patients thought to have died of liver insufficiency after a definite period of clinical disease as compared with a similar series of 197 cases in the control group. (See Table VII.)

The percentage of patients in each of the two groups dead at any given time after the onset of the initial symptoms is compared graphically in Fig. 1. No difference between the two groups of patients is apparent. The maximum

TABLE VII. SELECTION OF CASES FOR STUDY

	EXPERIMENTAL	CONTROL
Complicating disease	9	7
Inadequate data	21	62
Available for comparison	139	197
Total	169	266

known period of survival in the experimental group to date is slightly less than six years as compared with more than sixteen years in the control group.

A definite time (within a month) of onset of ascites was elicited in 100 instances in the experimental group and in 154 instances in the control group.

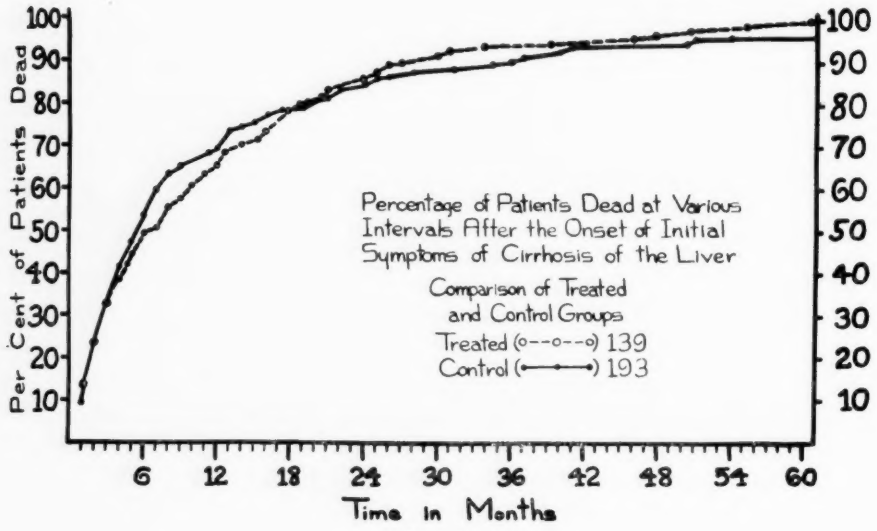

Fig. 1.

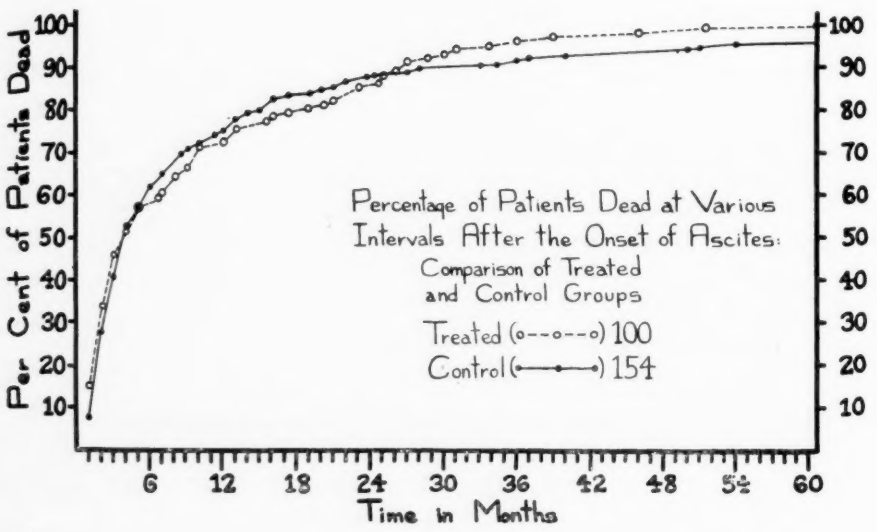

Fig. 2.

The percentage of patients dead at any given time after the onset of ascites is compared for the two groups in Fig. 2. The results are in no wise different from those described in the preceding paragraph.

A clear-cut history of the onset of jaundice, thought to be associated with the patient's cirrhosis, was elicited in forty-six experimental subjects and in sixty-three control patients. It is of interest that several patients gave a history of jaundice ten or more years prior to the obvious onset of cirrhosis.

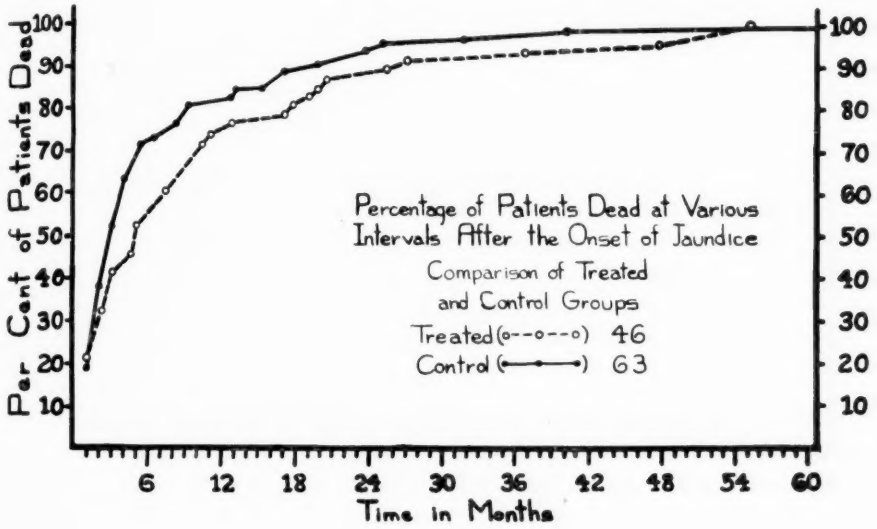

Fig. 3.

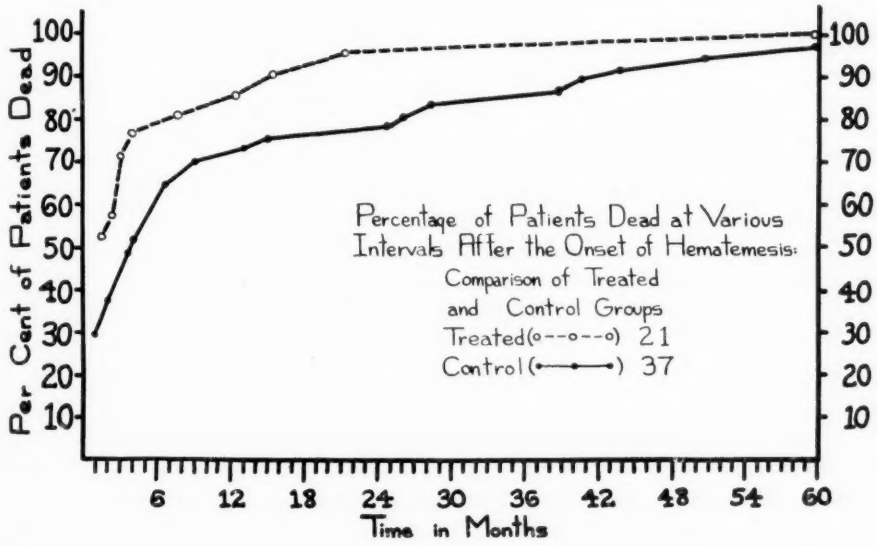

Fig. 4.

Such attacks of jaundice were not interpreted as heralding the onset of the patient's cirrhosis. The percentage of patients of the two groups of patients known to be dead at various times after the onset of jaundice is compared in Fig. 3. Again there is no basis for assuming any difference in the life expectancy of individuals in the two groups.

Finally, the percentage of patients dead at various intervals after the onset of gastrointestinal bleeding is compared in Fig. 4. The number of patients available for comparison was markedly less than in the previously described groups: twenty-one in the experimental group and thirty-seven in the control group. It will be noted that the treated group fared less well than the control group. Since more than half of the treated patients were dead in less than two months it is hardly reasonable to assume that therapy could have had any influence on these patients.

COMMENT

It is clear that in spite of preferential treatment with regard to supervision and follow-up care, the experimental group fared no better than the control group so far as survival is concerned. Several possible explanations for the failure of the therapeutic regimen to prolong life deserve consideration:

(1) The regimen was not adequately followed by the patients.
(2) The disease was so far advanced as not to be amenable to therapy.
(3) The therapeutic regimen was inadequate in one or more respects.
(4) Initial liver damage, possibly by factors other than dietary defects, had rendered the organ incapable of utilizing the materials supplied.

(1) Since 60 to 75 per cent of the patients in this series were chronic alcoholics it is probable that difficulty was encountered in enforcing the therapeutic regimen when the patients were not under observation in the hospital. In addition it is important to point out that many of these patients were without adequate resources for the purchase of food and other essentials. To avoid this difficulty, as previously indicated, prolonged hospitalization was utilized freely. Attempts at the evaluation of the adequacy of therapy in individual cases was made but was found to be impracticable.

(2) Evaluation of the stage of the patient's disease was also difficult. The size of the liver is ordinarily considered to decrease as the cirrhosis advances. This we found was not strictly true. A large percentage (70 to 75 per cent) of the patients studied, however, did have definite hepatic enlargement suggesting hypertrophy and/or fat infiltration. Such patients may reasonably be expected to respond more quickly and adequately than those with small, hard, intensely fibrosed livers.

(3) Analysis of the therapeutic regimen in the light of clinical and laboratory observations of the past few years suggests some desirable changes. The importance of supplying adequate amounts of an adequate variety of amino acids should be emphasized. The patient with cirrhosis is invariably suffering from depletion of tissue protein as well as blood protein. The keen competition for individual amino acids that may ensue under such circumstances by a multiplicity of body processes is well known. Fat transport and utilization as well as repair of damaged liver parenchyma are at the mercy of the amino acid supply.

The amino acid supply may be influenced profoundly by the presence of pancreatic disease, edema or atrophy of the intestinal tract, and the palatability

of the diet (fat content). These obstacles may be at least partially overcome by supplementing the oral consumption of protein with parenteral amino acids, at least at the onset of therapy.

Some doubt may be cast upon the wisdom of strict limitation of the fat intake. The relative importance of deficiencies of the essential fatty acids, excessive cholesterol intake, and adverse effect upon the protein intake cannot be decided readily. The great importance of protein would certainly argue in favor of considerably more freedom in the fat intake than has been permitted in this therapeutic experiment, so as to assure optimal protein intake.

Only a limited number of studies are available upon which one might base any accurate statements regarding choline requirements or tolerance in man. It is known that the requirement is influenced by other dietary factors, that is, protein (methionine, cystine), growth, and so forth. Assuming the requirement in man to be comparable to that in the dog, rat, or chick, it is reasonable to assume that the human requirement is near 1.5 to 3.0 Gm. daily.[1] The average diet of man contains 1.5 to 4.0 Gm. daily.[1] If the toxic effect of choline on man is comparable to that experienced by chicks and mice, man would be expected to experience minimal toxic effects from 15 to 20 Gm. of choline daily.[1] Assuming the daily diet of the patients studied to have contained 4.0 Gm. of choline, even with the maximum dose used (8 Gm.) no toxic manifestations would have been anticipated. It is possible that a marked increase in choline dosage is indicated since Kaplan and Chaikoff state that as much as 3 Gm. of choline daily are required to cure fatty livers in dogs if such fat infiltration is permitted to develop.[3] Upon the basis of body weight this is comparable to doses of 15 to 30 Gm. daily in a human being weighing 60 kilograms. Much of the animal experimentation has been preventive rather than curative and is of little value in determining the dose needed in the treatment of human beings with established liver lesions.

The methionine requirement is probably influenced by other dietary factors, growth, and so forth, as is the choline requirement. Very little specific information is available to guide one in the matter of dosage.

While the use of supplements of the B complex (brewers' yeast, crude liver extracts, and so on) was avoided in the treatment of the series of patients described, there can be no doubt that such supplements are indicated in view of the multiple evidences of malnutrition observed in patients with cirrhosis. Aside from the correction of multiple specific deficiencies, the salutory effect upon the patient's appetite is of extreme value. If was reasoned that mobilization of liver fat would be attended by improvement in appetite, and relief of specific deficiencies, by the ingestion of an adequate diet. This may be true but valuable time may be lost at a critical stage in the patient's illness.

(4) Primary damage to the liver may so impair its ability to discharge its responsibilities for fat metabolism that a fatty liver will ensue no matter how adequate the supply of dietary essentials may be. Such primary damage might be produced by many agents or methods. Experimentally, phosphorus or carbon tetrachloride may produce such impairment of function even though accompanied by or preceded by the use of lipotropic factors. If the influence of the

noxious agent is eliminated and enthusiastic therapy begun, the damage may be reversible. Unrecognized or inadequately treated damage of this sort in the human subject may be very difficult or impossible to treat successfully when it ultimately comes to the attention of the clinician.

CONCLUSIONS

Low fat diets supplemented by 1 to 8 Gm. of choline and one quart of skimmed milk daily (0.9 Gm. methionine) failed to influence the life span of patients with cirrhosis of the liver, even though these patients were much more closely supervised than the control series.

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DISTRIBUTION OF GOLD IN THE ANIMAL BODY IN RELATION TO ARTHRITIS

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ALTHOUGH gold and gold salts have had wide clinical application in diseases of the skin, in tuberculosis, and of late in rheumatoid arthritis, their mode of action is undetermined. Worth-while information about the pharmacology of gold salts has been gained particularly through the application of a micro method developed by Block and Buchanan[1] in the metabolic studies of Freyberg and associates[2, 7] as well as in the investigations by Denko and Anderson,[8] Cortell and Richards,[9] and Hartung and Cotter.[10] The following contains experimental data on the distribution of gold given as radioactive material and first used by Ely.[11]

METHOD

Au^{198} (half life, 2.7 days) produced in a pile by the (n, γ) reaction was obtained from the Atomic Energy Commission with a specific activity of about 2.5 mc. per milligram. Radioactive gold sodium thiosulfate was synthesized from the radioactive gold and administered intravenously. The tissue samples and excreta were weighed and digested in nitric acid and hydrogen peroxide; the gold was then quantitatively removed by a process of electrodeposition[12] developed by Dunn of this laboratory. The planchets with the radioactive gold were counted by means of a bell jar Geiger-Müller counter with a thin mica window. All data presented were corrected for half life.

RESULTS

The excretion of Au^{198} was studied in two white rats. The fractions of initially administered drug excreted during the first eight days are given in Table I. After the first week excretion became very slow. Further excretion studies are now in progress.

Gold Distribution in Rats.—Distribution experiments were set up using rats in which quantitative recovery of the administered gold was attempted. In these experiments all excreta were saved, the gold being recovered from this and from the whole carcass (minus samples removed). When such a procedure was followed, 97.75 per cent of the total gold administered could be recovered. Duplicate samples of each tissue were taken as a further check on the accuracy of the method employed.

The discrepancies indicated for bone and synovialis cannot be explained completely. In the case of synovialis, values tend to become higher at longer

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TABLE I

TIME (DAY)	GOLD THIOSULFATE EXCRETION (%)	
	URINE	FECES
1	8.4	3.5
2	2.7	1.0
3-4	2.8	0.8
5-6	1.1	0.6
7-8	1.0	0.8
Total in 8 days	16.3	6.7

Administered 1 mg. 0.1 millicurie.

TABLE II. DISTRIBUTION OF GOLD SODIUM THIOSULFATE IN THE RAT, QUANTITATIVE RECOVERY

TISSUE	PER CENT OF INJECTED DOSE PER GRAM TISSUE	
Muscle	.101	.078
Skin	.246	.236
Liver	.663	.660
Kidney	5.780	5.820
Spleen	1.310	1.180
Heart	.152	.187
Intestine	.220	.210
Lung	.131	.129
Synovialis	.295	.483
Articular cortex (bone)	.212	.257
Bone	.00057	.0014
Blood	3.87	3.47
Carcass		21.31
Blood		52.20
Excreta		24.24
Total recovered		97.75%

Duplicate samples used. Intestinal sample taken from ileum. Administered dose, 1.2 mc. 1 mg. gold). Rat sacrificed forty-eight hours after administration.

Articular cortex refers to cartilage of joint surface and compact bone directly under it.

intervals, and there is also the factor of small sampling. Synovial samples from rats average 5 to 10 mg., and small amounts of evaporation could easily account for significant weight loss in such samples. Any such error is greatly increased when data are expressed in terms of grams.

When the accuracy of the technique was ascertained by such quantitative recoveries a series of fifteen rats was employed, each rat receiving 2 ml. of radiogold sodium thiosulfate solution containing 1.0 mg. of gold and approximately 1 mc. of radioactivity. The amount of radioactivity varied with the time of injection and was in all cases exactly known. The animals were sacrificed at intervals of two to eight days. Before sacrifice these animals were perfused with physiologic saline solution through the proximal end of the inferior vena cava and were bled through the distal end of the same vessel; in some cases, however, the saline was introduced directly into the heart while bleeding took place through the inferior vena cava.

The agreement between respective determinations was fair in most cases; however, some organs showed considerable fluctuations in gold content—notably the kidney and spleen. The time interval had little to do with the values of

any given tissue, which would seem to indicate that gold is held rather firmly by the respective tissues once it has left the blood stream. As can be seen in Table I, the amount excreted is small after the fourth day.

Gold distribution data were taken on ten rabbits at different time intervals. Before sacrifice the rabbits were perfused with normal saline through the proximal end of the inferior vena cava and bled through the distal end of the same vessel. There was a general agreement between respective determinations although some organs showed considerable fluctuations in gold content. A typical set of the results obtained for the concentration of Au^{198} in various types of tissue is given in Table IV.

TABLE III. DISTRIBUTION OF GOLD IN RATS

Tissue	PER CENT OF INJECTED DOSE PER GRAM TISSUE
Kidney	8.72
Spleen	1.14
Thyroid	.78
Synovialis	.67
Liver	.67
Tendon	.52
Skin	.47
Articular cortex (bone)	.45
Testicle	.28
Heart	.21
Muscle	.07

These data represent the averages on fifteen rats.

TABLE IV. GOLD DISTRIBUTION IN THE RABBIT; DOSE 404 MICROCURIE AU (1.0 MG. AU); SACRIFICED SEVENTEEN DAYS AFTER ADMINISTRATION

TISSUE	PER CENT OF INJECTED DOSE PER GRAM TISSUE
Kidney	.37
Spleen	.14
Liver	.01
Adrenal	.03
Tendon	.023
Skin	.016
Small intestine	.015
Synovialis	.014
Bone	.014
Blood	.014
Marrow	.013
Lymph node	.012
Aorta	.012
Testicle	.01
Heart	.009
Articular cortex (bone)	.007
Lung	.006
Muscle	.002

In the course of this work specific activities of several tissues were ascertained for which the uptake of gold salts had not been demonstrated previously, such as the iris, aqueous and vitreous humors, and brain. All these organs contained gold quantities in the order of 10^{-4} per cent of the injected amount per gram of tissue.

A comparison of the data given for rabbits and rats shows great variance which is due to the greatly increased dilution of the administered dose in the rabbit. The average weight of the rats used was 150 grams, while that of the rabbits equaled 2.5 kilograms. When correction was made for this factor the results were comparable, as can be seen in Table V.

TABLE V. COMPARISON OF GOLD DISTRIBUTION IN RABBITS AND RATS

TISSUE	RAT	RABBIT
Kidney	8.72	6.14
Spleen	1.14	2.3
Liver	.67	1.16
Synovialis	.67	.23
Tendon	.52	.38
Skin	.47	.28
Articular cortex	.45	.12
Heart	.21	.15
Muscle	.07	.03

Values expressed in per cent total dose injected per gram tissue.

The similar relative order is interrupted by the synovial sample in the rat, which may be falsely high.

Gold Uptake in Chemical Synovitis.—Chemical arthritis was produced in five rabbits by the intra-articular injection of 0.5 c.c. of a solution, composed of U. S. P. turpentine 3 parts and diethylether 1 part, into the knee joint of one extremity. This was repeated in five days. Ten days after the initial injection the animals were given the radiogold thiosulfate by the intravenous route, and five days later the rabbits were sacrificed for assay after first being perfused with 0.89 per cent saline. The rabbits had not been walking since twenty-four hours following the initial treatment. The joints were swollen to twice the normal size and were tender and hot to the touch.

On autopsy the synovialis showed proliferation to a moderate degree and the articular surfaces were dull and roughened.

As can be seen from Table VI, in all cases the chemically inflamed tissues took up a significantly larger amount of gold salt than the normal tissue. From previous experience with the distribution of gold in the blood components it was known that the white blood cells contained considerable amounts of thiosulfate, and it was thought that this fact might account for the higher level in

TABLE VI. CHEMICAL ARTHRITIS

N-Tendon	.004	.006	.028	.023	.026
P-Tendon	.013	.016	.124	.27	.04
N-Synovia	.015	.018	.074	.16	.002
P-Synovia	.058	.084	.225	—	.093
N-Cortex	.009	.018	.05	.13	.05
P-Cortex	.02	.05	.085	.19	.11
N-Muscle	.0029	.07			
P-Muscle	.03	.113			
PUS	.028	.033			.05

Rabbits were given 1.5 mc. ten days after turpentine injection. Values are expressed in per cent total dose per gram tissue.

P and N refer to pathologic and normal respectively.

the inflamed tissues; however, pure pus from sterile abscesses was found to have less activity per gram than the tissues under consideration, and in tissues such as bone and tendon large exudative responses were not noted. It was clear from this that even though the inflammatory elements might be responsible for part of the concentration they could not possibly account for all and that in the presence of inflammation the tissues themselves soaked up more of this salt.

In view of these facts we wondered about the specificity of this phenomena for joint structures. It was found that apparently the reaction would occur outside of joints, for when the same chemical irritant was injected into muscle and a sterile abscess formed, the muscle wall of the abscess would also accumulate the salt. This perhaps makes the accumulation in the joints less noteworthy but nonetheless real.

Human Experimentation.—It was possible* to carry out a preliminary distribution experiment on a human arthritic subject. The patient selected was a 57-year-old white woman with rheumatoid arthritis of a moderately advanced stage and of approximately ten years' duration. Pain upon motion was present in both knees; movement was only slightly restricted. The patient had never received chrysotherapy. She was given 1,010 microcuries of radiogold as the sodium thiosulfate salt, representing 2.5 mg. of gold. Twenty-four hours after administration, biopsy of the left knee was done†; specimens of skin, superficial fascia and fat, deep fascia and fat, synovial fluid, synovial membrane, and muscle were taken. Of the tissues taken, the synovialis was by far the most active, with the synovial fluid next in activity. This indicates that in human beings with rheumatoid arthritis the synovialis is at least somewhat of a concentrator of gold.

TABLE VII. GOLD DISTRIBUTION IN RHEUMATOID ARTHRITIS

Synovialis	.014
Synovial fluid	.0094
Muscle	.0013
Superficial fascia	.000366
Deep fascia	.000596
Skin	.00077

It is interesting to note that the synovialis is eighteen times higher in gold content than skin. If we compare the gold concentrating ability of human and rat skin, the value (.014 per cent) for synovialis is in the range one would expect for kidney—18 times .47 (rat skin) equals 8.46 (value for rat kidney).

CONCLUSIONS

Au^{198} is a suitable radioactive isotope for studying the action of gold on the animal body. Excretion and distribution data obtained with this isotope

*Through the cooperation of Professor William J. Kerr, Chairman, Department of Medicine, University of California Medical School.

†By Dr. Verne Inman, Department of Surgery, University of California Medical School.

conform with previous observations using stable gold. Synovialis, tendon, and articular cortex in chemical arthritis show a greater uptake of gold than similar tissues of normal joints, and this was shown to be nonspecific since the muscle wall of sterile abscesses exhibits the same phenomenon. Passage of gold from the blood stream into the central nervous system and ocular structures of rabbits has been demonstrated.

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THE EFFECT OF NUTRITION ON THE TUMOR RESPONSE IN ROUS CHICKEN SARCOMA

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INTRODUCTION

NUMEROUS observations have confirmed the fact that nutrition may influence the genesis and growth of tumors in rats and mice. The search for requirements in the nutrition of malignant tissue should reveal findings that are valid regardless of the nature of the tumor studied or the kind of animal used, as well as more limited findings. Such information might well include the facts that adequate caloric intake has been shown necessary for tumor growth[1] and that vitamins such as pantothenic acid and riboflavin have been found stimulative,[2] whereas choline may be inhibitory.[3]

EFFECT OF NUTRITION IN ROUS SARCOMA

Little and co-workers[4] showed that folic acid has a marked effect on the tumor response in Rous chicken sarcoma. The effectiveness of folic acid free diets in preventing the tumor response of baby chicks and the rapidity and regularity with which the response occurred in adequately nourished chicks led us to our present study of the effect of each of the different constituents in a synthetic diet for chickens.

The data to be presented confirm observations made on rats and mice to the effect that malignant tissue may utilize vitamins such as pantothenic acid and riboflavin for growth. Our results also indicate that nicotinamide and cholic acid may be stimulative. However, folic acid appears to be the only nutrient required to the extent that tumor response is prevented by its absence.

Method for Demonstrating the Effect of Nutrition.—In our tests, the extent to which each nutrient influenced tumor response was determined by comparing the incidence of tumors in groups of chicks fed the complete diet with the incidence of tumors in groups fed the same diet without the nutrient. By comparing observations made on the eighth, tenth, twelfth, fourteenth, and sixteenth day, the effect of nutrition was evaluated for all stages from the usual time of first appearance of tumors to the time when 90 to 100 per cent of control birds showed tumors. The response with and without nutrient was calculated from the combined results of different tests where more than one test was carried out. The percentage of birds showing tumors when fed diets with the nutrient was divided by the percentage showing tumors when fed the same diet without the nutrient. The nutrients which showed no stimulative effect gave values of approximately 1.0.

Relation of Size of Inoculum to Tumor Response.—An even distribution of tumor responses over the eight days selected for observation of results was readily obtained by controlling the size of the inoculum. As shown in Table I, there is a direct correlation between the size of inoculum and the length of the latent period.

We used homogeneous samples of frozen virus, prepared by blending fresh tumor tissue in a Waring mixer and weighing 2 Gm. amounts into sterile Petri plates to be stored in a dry

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TABLE I. TUMOR RESPONSE OBTAINED WITH HOMOGENEOUS PREPARATION OF INOCULUM KEPT FROZEN FOR VARIOUS PERIODS

AMOUNT OF INOCULUM USED (0.25 ML. DOSE)	INOCULUM KEPT FROZEN (DAYS)	TOTAL NUMBER OF CHICKS USED	NUMBER OF CHICKS THAT GREW TUMORS	PERCENTAGE OF TAKES	AVERAGE LATENT PERIOD (DAYS)
10 mg.	Fresh	21	21	100.0	7.90
	16	16	15	93.7	7.93
1 mg.	Fresh	25	25	100.0	9.56
	16	16	15	93.7	9.66
	24	190	173	90.1	9.53
0.1 mg.	Fresh	25	23	92.0	12.04
	16	17	16	94.1	11.25
	24	190	157	82.6	11.08
0.01 mg.	Fresh	23	13	56.5	12.84
	16	20	8	40.0	14.00

ice chest until used. Preliminary suspensions containing 10 mg. in a 0.25 ml. dose were prepared by adding 50 ml. of 2 per cent peptone solution to 2 Gm. of tumor tissue. A Ten Brock grinder was employed, and further dilutions were made in peptone solution.

In our experiments the desired length of latent period was obtained with 1 mg. of inoculum. The frozen virus was found to be stable for at least a month. It is of interest that Reinhard and co-workers[5] found a similar relationship between the size of inoculum and the length of the latent period for growth of a transplantable mouse adenocarcinoma.

One-day-old New Hampshire Red chicks which were the progeny of a selected flock (as described in our previous report[4]) were injected in the right breast with 0.25 ml. of the suspension containing the 1 mg. dose. The feathers were removed from the breast area before inoculation. Groups of ten birds were placed on diets with and without each nutrient tested. Nutrients which were of interest because of possible stimulative effect were retested several times. The chicks were maintained in electric brooders at 90° F.; water and food were supplied ad libitum. Approximately 400 chicks (ten to twenty per group) were used in each experiment.

The basal diet contained 53 per cent Cerelose,* 22 per cent alcohol extracted casein, 4.3 per cent salt mixture, 3 per cent calcium gluconate, 8 per cent gelatin, 4 per cent Ruffex,† 5 per cent soybean oil, 0.25 per cent cholic acid, 0.45 per cent cystine, 200 mg. per cent choline chloride, 3 mg. per cent calcium pantothenate, 3 mg. per cent nicotinamide, 0.5 mg. per cent pyridoxine, 0.3 mg. per cent thiamin chloride, 0.03 mg. per cent biotin, 0.5 mg. per cent riboflavin, 100 mg. per cent inositol, 5 mg. per cent para-aminobenzoic acid, 0.2 mg. per cent folic acid, 5 mg. per cent vitamin E, 0.2 mg. per cent vitamin K, 3,500 units per cent vitamin A, and 200 units per cent vitamin D. The diet with nutrient was usually the complete diet. A commercial chick starter gave the same tumor response as the complete synthetic diet. In studies of substitutes for soybean oil, the diet with nutrient contained the substitute and the basal diet was soybean oil free. In these tests the vitamins A, D, E, and K were added in propylene glycol.

The chicks were observed daily beginning with the eighth day and continuing through the sixteenth day at which time adequately nourished groups showed 90 to 100 per cent tumors. Wing tags applied on the sixth or seventh day were used to identify individual birds. The increasing sizes of tumor were recorded (as previously described) for evidence as to the accuracy of first observations. Tumors recorded as questionable were counted in determining the response whenever this observation was confirmed by subsequent findings.

*Glucose monohydrate. Fisher Scientific Co., Pittsburgh, Pa.

†Purified cellulose containing 70 per cent α cellulose and 30 per cent other celluloses. Fisher Scientific Co., Pittsburgh, Pa.

EXPERIMENTAL RESULTS

Three distinct types of results have been obtained with nutrients which demonstrated a definite stimulative action on tumor growth. While with and without values of approximately 1.0 were obtained in tests on nutrients which did not stimulate tumor growth, (A) constant values of approximately 3.0 were obtained in tests of riboflavin, (B) descending values of 13.0, 6.2, 4.6, 2.6, 2.1 were obtained in tests of nicotinamide, and (C) ascending values of 13.0, 40.0, 63.0, 89.0, 93.0 were obtained in tests of folic acid. While other nutrients gave results similar to those illustrated in A and B, folic acid was the only nutrient giving the result C.

The Effect of Vitamins From Liver.—Table II shows the effect of nine water-soluble vitamins on the tumor response of chicks to Rous sarcoma virus. Folic acid produced the greatest effect and para-aminobenzoic acid and biotin produced the least effect. Nicotinamide and calcium pantothenate influenced the rate of growth more than the final incidence; riboflavin did not change the rate of growth but did influence the incidence at all stages.

TABLE II. EFFECT OF WATER-SOLUBLE VITAMINS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

VITAMIN	AMOUNT IN DIET	NUMBER OF CHICKS (A/B†)	NUMBER OF TESTS	RESPONSE WITH VITAMIN / RESPONSE WITHOUT VITAMIN				
				8	10	12	14	16
Thiamin*	3 mg./kg.	10/10	1	30.0	1.5	1.3	1.1	1.1
Riboflavin	5 mg./kg.	39/37	4	2.1	3.0	3.1	3.1	3.1
Pyridoxine*	5 mg./kg.	30/30	3	2.0	2.8	1.8	1.4	1.3
Nicotinamide	30 mg./kg.	29/29	3	13.0	6.2	4.6	2.6	2.1
Calcium pantothenate*	30 mg./kg.	29/18	3	24.0	11.0	2.6	2.0	1.8
Inositol	1 Gm./kg.	29/25	3	13.0	1.2	1.1	1.0	1.0
Para-aminobenzoic acid	50 mg./kg.	19/19	2	1.4	1.1	1.0	0.8	0.8
Biotin	0.3 mg./kg.	29/27	3	1.1	1.8	1.2	1.1	1.1
Folic acid	2 mg./kg.	30/32	3	13.0	40.0	63.0	89.0	93.0

*Vitamin deficient chicks were revived with complete diet beginning on the tenth day.
†a/b, Total chicks used to determine effect of diets (a) with and (b) without the vitamin specified.

Since deficiencies of thiamin, pyridoxine, and calcium pantothenate caused serious loss of weight, chicks fed diets without these vitamins were revived with complete diet beginning on the tenth day. In previous work with folic acid it had been shown that the stimulative effect of restoring this vitamin on the tenth day does not become apparent for at least seven days. This practice was adopted in the case of thiamin, pyridoxine, and calcium pantothenate deficiencies to permit survival of the birds for the duration of the test. The responses with and without one of these vitamins for the twelfth, fourteenth, and sixteenth day may or may not be influenced by restoring the vitamin on the tenth day.

Since liver is a rich source of still unidentified vitamins, several fractions of liver were tested in the presence of the nine purified vitamins for possible stimulative effect on the tumor response of chicks to Rous sarcoma virus. None of the liver fractions influenced the rate of growth or incidence of tumor to an extent which would indicate the presence of additional factors for tumor growth.

The Effect of Vitamins A, D, E, and K.—The results of tests of diets with and without vitamins A, D, E, and K are shown in Table III. None of these vitamins stimulated tumor growth when present in the diet. The values 0.8, 0.6, and 0.5 suggest that the oil-soluble vitamins may slightly retard tumor growth. It is of interest that diets with and without the combination of soybean oil and vitamins A, D, E, and K influenced the tumor response to a greater extent than did the diets with and/or without the oil-soluble vitamins alone.

TABLE III. EFFECT OF OIL-SOLUBLE VITAMINS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

VITAMIN	AMOUNT IN DIET	NUMBER OF CHICKS (A/B*)	NUMBER OF TESTS	RESPONSE WITH VITAMIN / RESPONSE WITHOUT VITAMIN 8	10	12	14	16
Combined								
A	35,000 I.U./kg.							
D	2,000 I.U./kg.	19/19	2	0.8	1.0	1.0	1.0	1.0
Combined								
A	35,000 I.U./kg.							
D	2,000 I.U./kg.							
E	50 mg./kg.							
K	2 mg./kg.	10/10	1	5.0	1.3	1.1	1.1	1.1
E	50 mg./kg.	10/ 8	1	0.6	1.2	1.4	1.2	1.2
K	2 mg./kg.	10/10	1	0.5	1.0	1.5	1.1	1.0
Combined								
Soybean oil	5 per cent							
A, D, E, K	As above	20/23	2	10.0	2.6	2.3	2.0	1.6

*A/B, Total chicks used to determine effect of diets (A) with and (B) without the vitamin specified.

The Effect of Various Fats and Oils.—Table IV shows the results of tests in which twelve different substances were tested in a soybean oil free diet. None of these substances stimulated tumor growth. Soybean lecithin, cod-liver oil, and linoleic acid appeared to retard tumor growth when present in the diet. Most of the other substances in the group showed this effect to some degree.

TABLE IV. EFFECT OF FATS AND OILS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

NUTRIENT*	AMOUNT IN DIET (%)	NUMBER OF CHICKS (A/B†)	NUMBER OF TESTS	RESPONSE WITH NUTRIENT / RESPONSE WITHOUT NUTRIENT 8	10	12	14	16
Beef liver fat	5	10/10	1	1.0	1.0	0.7	1.0	1.0
Cholesterol	1	54/54	3	1.0	1.2	0.7	0.7	0.8
Coconut oil	5	30/30	3	0.1	1.3	1.1	1.3	1.3
Cod-liver oil	3	10/10	1	1.0	0.7	0.4	0.5	0.6
Corn oil	5	10/10	1	0.5	0.5	0.8	1.0	1.0
Crisco	5	10/10	1	1.0	1.2	0.5	0.8	0.8
Lanolin	3	10/10	1	1.0	0.7	0.5	0.8	0.8
Lard	5	10/10	1	0.0	0.7	0.5	0.6	0.8
Linoleic acid	3	30/30	3	0.0	0.5	0.7	0.8	0.8
Sodium oleate	3	30/30	3	0.1	1.2	1.0	1.2	1.3
Soybean lecithin	1	20/20	2	0.0	0.8	1.3	1.4	1.3
Soybean lecithin	3	10/10	1	0.0	0.5	0.5	0.6	0.6
Soybean oil	5	20/20	2	2.0	1.3	1.0	1.0	1.0

*The basal diet was oil-free; vitamins A, D, E and K were added in propylene glycol.

†a/b, Total chicks used to determine effect of diets (a) with and (b) without the substance specified.

The Effect of Cholic Acid.—Table V shows the results of tests of diets with and without cholic acid, sodium chloride, gelatin, calcium gluconate, and Ruffex. Diets with and without cholic acid influenced tumor growth in much the same way as did diets with and without riboflavin. Three times as many tumors developed when cholic acid was present in the diet.

TABLE V. EFFECT OF CHOLIC ACID IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

NUTRIENT	AMOUNT IN DIET (%)	NUMBER OF CHICKS (A/B*)	NUMBER OF TESTS	RESPONSE WITH NUTRIENT / RESPONSE WITHOUT NUTRIENT				
				8	10	12	14	16
Cholic acid	0.25	20/20	2	30.0	3.2	3.8	3.1	3.1
Sodium chloride	1	15/15	1	0.4	0.6	0.9	0.8	0.9
Gelatin	8	10/10	1	3.0	2.3	1.4	1.2	1.2
Combined								
Gelatin	8							
Calcium gluconate	3	10/10	1	3.0	7.0	1.6	1.6	1.4
Ruffex	4	65/65	4	1.6	1.0	0.9	0.8	0.8

*a/b, Total chicks used to determine effect of diets (a) with an (b) without the substance specified.

SUMMARY

The effect of nutrition on the tumor response in Rous chicken sarcoma was determined by comparing observations of chicks fed synthetic diets with and without each nutrient. Tumor response was stimulated by the presence of folic acid, nicotinamide, calcium pantothenate, riboflavin, and cholic acid in the diet. Folic acid was the only nutrient required to the extent that tumor response was prevented by its absence from the diet.

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THE USE OF ANTAGONISTS OF PTEROYLGLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

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SINCE Woods[1] described the interference of *p*-aminobenzoic acid on the action of sulfonamides, the antagonism between compounds promoting growth and those which inhibit growth has received increasing attention. Analogues of pteroylglutamic acid (folic acid) have been studied extensively.[2] The antagonistic activity of these compounds in the growth of *Streptococcus faecalis R* has been examined by Hutchings and Stokstad.* Experiments leading to the demonstration of a stimulatory effect of pteroylglutamic acid and an inhibitory effect of its antagonists on Rous chicken sarcoma have been reported by Little and co-workers[4] and by Woll.[5]

In the present report we wish to present more recent findings with regard to the effect of choice of antagonists and method of administration on the outcome of experiments with Rous chicken sarcoma. Table I contains a list of the antagonists of pteroylglutamic acid which we have tested for ability to control Rous chicken sarcoma. Both the abbreviated names of compounds (such as used in our first report on Rous sarcoma) and the complete chemical names are shown as an aid in referring to reports dealing with the synthesis of these compounds. The antagonists which we have found most useful, for reasons to be discussed, are the 4-amino-pteroylaspartic acid and the 4-amino-pteroyl d(-)glutamic acid which were synthesized by Mowat and others,[10*] and the 4-amino-pteroylglutamic acid synthesized by Seeger, Smith, and Hultquist.[7†]

PROCEDURE

In producing Rous chicken sarcoma we have employed homogeneous samples of frozen virus prepared by blending fresh tumor tissue in a Waring mixer and weighing 2 Gm. amounts into sterile Petri plates to be stored in a dry ice chest until used. Preliminary suspensions containing 10 mg. in a 0.25 ml. dose were prepared by adding 50 ml. of 2 per cent peptone solution to 2 Gm. of tumor tissue. A Ten Brock grinder was employed, and further dilutions were made in peptone solution. The size of inoculum used in our experiments was determined by titration of the virus for even distribution of tumor responses over a period of time from the eighth to the sixteenth day of the test. The frozen virus was found to be stable for at least a month with respect to the effect of concentration of virus on the time distribution of tumor responses. Table II illustrates the method employed for standardizing the virus.

HANDLING OF CHICKS

New Hampshire Red chicks which were the progeny of a selected flock (as described in our previous report)[4] were injected in the right breast with 0.25 ml. of the suspension containing the dose required for the desired distribution of tumor responses. The feathers were removed from the breast area before inoculation. Groups of ten birds were compared with

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*Lederle Laboratories Division.
†Calco Chemical Division.

TABLE I. LIST OF ANTAGONISTS OF PTEROYLGLUTAMIC ACID TESTED ON ROUS CHICKEN SARCOMA

ABBREVIATED NAME	CHEMICAL NAME	REFERENCE
Pteroylaspartic acids*	*N*-[4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoyl]-aspartic acid	6
4-Amino-folic acid* or 4-Amino-pteroylglutamic acid	*N*-[4-{[(2,4-diamino-6-pteridyl)-methyl]-amino}-benzoyl]-glutamic acid	3, 7
N-methyl-folic acid* or *N*-methyl-pteroylglutamic acid	*N*-[4-{*N*[(2-amino-4-hydroxy-6-pteridyl)-methyl]-*N*-methylamino}-benzoyl]-glutamic acid	3, 8
N-methyl-pteroic acid*	4-{*N*-[(2-amino-4-hydroxy-6-pteridyl)-methyl]-*N*-methylamino}-benzoic acid	3, 8
4-Amino-*N*-methyl-folic acid* or 4-Amino-*N*-methyl-pteroylglutamic acid	*N*-[4-{*N*-[(2,4-diamino-6-pteridyl)-methyl]-*N*-methylamino}-benzoyl]-glutamic acid	3, 9
4-Amino-*N*-methyl-pteroic acid*	4-{*N*-[(2,4-diamino-6-pteridyl)methyl]-*N*-methylamino}-benzoic acid	9
4-Amino-pteroylaspartic acid*	*N*-[4-{[(2,4-diamino-6-pteridyl)-methyl]-amino}-benzoyl]-aspartic acid	10
4-Amino-folic acid with d(-)glutamic acid* or 4-Amino-pteroyl-d(-)glutamic acid	*N*-[4-{[2,4-diamino-6-pteridyl)-methyl]-amino}-benzoyl]-d(-)glutamic acid	10

*Designation used in our first report on the effect of folic acid and its antagonists on Rous chicken sarcoma.[4]

TABLE II. TITRATION OF ROUS SARCOMA VIRUS FOR EVEN DISTRIBUTION OF TUMOR RESPONSES

AMOUNT OF TISSUE IN INOCULUM (0.25 ML.)	TOTAL CHICKS USED	TOTAL TESTS MADE	PERCENTAGE OF CHICKS WITH TUMORS				
			DAY				
			8	10	12	14	16
10 mg.	37	4	64	83	90	90	97
1 mg.*	140	14	14	40	58	71	91
100 μg	140	14	0	6	26	45	71
10 μg	140	14	0	1	3	14	31

*This amount of virus gave the desired distribution of tumor responses.

as many untreated controls. The chicks were maintained in electric brooders at 90° F.; water and food were supplied ad libitum. Baby chicks varying from 1 to 8 days old were tested for ability to resist the toxic effect of different antagonists of folic acid.

A daily record of observations was made from the eighth day of the test to the twentieth day. The growth of tumors from the time of first appearance was recorded in the manner previously described. This information enabled us to demonstrate retardation of tumor growth when inadequate doses of antagonists were used. In the tables presented in this paper, chicks showing the slightest evidence of tumor have been counted in with those showing the usual tumor response characteristic of untreated groups.

EXPERIMENTAL RESULTS

Table III shows the results of experiments with 4-amino-pteroylaspartic acid. This antagonist of folic acid was administered (1) by daily intraperitoneal injection to chicks which were 2 days old at the start, (2) by daily intraperitoneal injection to chicks which were 8 days old at the start, and (3) by feeding to chicks which were only 1 day old at the start. It is apparent from Table III that under the conditions in (1) the toxicity of the chemical for the chicks interfered with its value as an inhibitor of tumor growth. Under the conditions in (2) the chicks were more resistant to the toxic effect of doses inhibitory to tumor growth. Under the conditions in (3), chicks only 1 day old at the start could

TABLE III. USE OF 4-AMINO-PTEROYLASPARTIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG.)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks					
2	0.02	10	0	44	0
2	0.1	10	40	50	10
2	0.2	20	75	80	5
2	0.2	10	80	50	0
2	0.2	10	60	50	0
(2) Antagonist Administered by Daily Intraperitoneal Injection to Week-Old Chicks					
8	0.2	10	0	60	10
8	0.4	10	10	44	10
(3) Antagonist Administered in Diet of Baby Chicks					
1	20/kg.	15	0	0	9
1	80/kg.	20	10	75	5

TABLE IV. USE OF 4-AMINO-PTEROYL-D(-)GLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG.)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks					
3	0.01	10	0	22	0
2	0.02	10	20	42	0
3	0.1	10	30	75	0
2	0.2	20	95	66	5
2	0.2	10	80	50	0
(2) Antagonist Administered by Daily Intraperitoneal Injection to Week-Old Chicks					
8	0.1	10	20	37	10
8	0.2	10	30	42	10
8	0.4	10	60	100	10
(3) Antagonist Administered in Diet of Baby Chicks					
1	20/kg.	15	0	0	9
1	80/kg.	20	0	55	5

TABLE V. USE OF 4-AMINO-PTEROYLGLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START	DOSE OF CHEMICAL (MG.)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks					
2 days	0.01	10	100	—	0
3 days	0.1	10	100	—	0
(2) Antagonist Administered in Diet of Baby Chicks					
1 day	20/kg.	20	100	—	9
(3) Antagonist Administered by Daily Intraperitoneal Injection to Adult Birds					
6 weeks	1.0	10	10	62	0
7 weeks	1.0	10	0	60	0
8 weeks	1.0	10	0	40	0

be treated successfully with 80 mg. of 4-amino-pteroylaspartic acid per kilogram of diet without toxic effect. The diet was the regular commercial chick ration.

Table IV shows that similar results were obtained with 4-amino-pteroyl-d(-)glutamic acid. Daily injections of the doses of chemical required to inhibit tumor growth resulted in impairment of health and eventual loss of birds when the chicks were 2 to 3 days old at the start. Chicks 8 days old at the start showed greater resistance to the toxic effects of doses inhibitory to tumor growth. Chicks only 1 day old at the start showed no toxic effect when fed 80 mg. of 4-amino-pteroyl-d(-)glutamic acid per kilogram of diet.

Table V shows that 4-amino-pteroylglutamic acid (4-amino-folic acid)[4] is suitable for use in the treatment of adult birds only. In a series of experiments we succeeded in neutralizing the toxicity of 4-amino-pteroylglutamic acid for baby chicks by giving simultaneous injections of pteroylglutamic, pteroyldiglutamic, or pteroyltriglutamic acid. It was found that 0.25 mg. doses of pteroylglutamic acid protected 60 per cent of chicks, 0.25 mg. doses of pteroyltriglutamic acid protected 50 per cent of chicks, and 0.25 mg. doses of pteroyldiglutamic acid protected 10 per cent of chicks against the toxic effect of 0.01 mg. doses of 4-amino-pteroylglutamic acid. This antagonist did not prevent tumor growth in baby chicks thus protected from its toxic effect.

TABLE VI. USE OF 4-AMINO-*N*-METHYL-PTEROYLGLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG.)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks					
2	0.02	10	0	22	0
2	0.1	10	80	50	10
2	0.2	10	70	33	0
2	0.2	20	55	77	5

Table VI shows the results of experiments with 4-amino-N-methyl-pteroylglutamic acid. When administered to baby chicks by daily intraperitoneal injection, this antagonist demonstrated toxicity and also inhibited tumor growth. It was not administered in the diet because of lack of material. The toxicity of 4-amino-*N*-methyl-pteroylglutamic acid appears to be similar to that of 4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid. These antagonists, when injected, produce symptoms resembling those caused by maintaining chicks on folic acid free diets. We have found that chicks showing severe symptoms as a result of injection with these antagonists quickly revive when treatment is discontinued.

DISCUSSION

It is evident from our experiments that Rous chicken sarcoma may be controlled by regulating the amount of pteroylglutamic acid in the tissues of the chicken, either by use of synthetic folic acid free diets[4] or by use of suitable

chemical antagonists. Severe deficiencies of the vitamin, whether caused by lack of folic acid in the diet or by the injection of powerful antagonists such as 4-amino-pteroylglutamic acid, regularly result in death of birds. It is well known that the amount of folic acid required to maintain the normal health of chickens is relatively small. According to Oleson[11] 0.5 mg. per kilogram of diet is suboptimum, 1 to 2 mg. are optimum, and 2 to 5 mg. are superoptimum.

Our experiments indicate that at least two of the chemical antagonists of folic acid (4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid) and possibly a third (4-amino-*N*-methyl-pteroylglutamic acid) may be appropriate tools for controlling the amount of folic acid available in the body for tumor growth without depriving the body of the amounts of vitamin required for normal health. When the treated animal is a rapidly growing chick, these chemicals appear to be more serviceable than 4-amino-pteroylglutamic acid. When the treated animal is an adult chicken, 4-amino-pteroylglutamic acid is not only serviceable, but may well be the chemical of choice inasmuch as it is effective in small doses.

The toxicity of these chemicals appears to be due solely to the severity of the vitamin deficiency which may result from too intensive treatment. Our experiments indicate that treatment by mouth is the method of choice, since tumor growth may be inhibited by this method in a greater percentage of animals without toxic effect. Evidently the tumors of Rous Chicken sarcoma are more directly dependent for their growth on the folic acid furnished by the diet than are the normal tissues of the chick. Since chicks do not develop severe deficiencies of the vitamin in the time required to demonstrate inhibition of tumor growth by use of folic acid free diets (as shown in our previous report),[4] it is possible that the health of such chicks is protected by stores of the vitamin which are not available to tumors of this type. If such is the case, the most desirable method of treatment would be the administration in the diet of chemicals which would antagonize the vitamin present in food. We have shown that 4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid can produce this result.

SUMMARY

Two chemical antagonists of pteroylglutamic acid (4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid) have been found capable of inhibiting tumor growth in baby chicks inoculated with Rous sarcoma virus when the chemicals were fed ad libitum at a concentration of 80 mg. per kilogram of diet. These chemicals and also 4-amino-*N*-methyl-pteroylglutamic acid were active in inhibiting tumor growth in baby chicks when injected. Toxic effects resembling the effect of severe vitamin deficiency occurred when the chemicals were injected, but not when the chemicals were fed.

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TREATMENT OF THE TYPHOID CARRIER STATE

Trial of Two Chemotherapeutic Procedures

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ALTHOUGH the medical literature contains many reports of unsuccessful attempts to clear up the chronic typhoid carrier state by the use of chemotherapeutic agents, it nevertheless is deemed worth while to record the results of the two studies presented here since they represent failures to confirm previously published favorable reports.

Although typhoid fever in New York State exclusive of New York City is rapidly reaching the vanishing point (fifty-two cases reported in 1947), the residual chronic carriers living in the state continue to offer the threat of further outbreaks of the disease. A total of 465 known chronic carriers are listed on the roster of the New York State Department of Health, and it is estimated that some 2,500 carriers actually survive in the state at the present time.[1] A chemotherapeutic agent effective in clearing up the typhoid carrier state would thus be a real boon to public health as well as to the individual carriers involved.

Trial of Tin Compound in the Treatment of Typhoid Carriers.—The report by Reitler and Marberg[2] presenting apparently clear-cut evidence of therapeutic benefits afforded by a tin compound (heptadekylaldehyde stannoxysterate) in the treatment of typhoid fever and, more specifically, in the cure of two typhoid carriers seemed to warrant further clinical trial of the compound. Supplemental information[3] indicated that three of four definitely proved chronic typhoid carriers were cleared of their carrier condition by administration of this drug. Accordingly in December, 1944, twenty-one chronic typhoid carriers, inmates of two New York State mental hospitals,* were selected for treatment. Preliminary stool examinations† of the carriers established the persistence and constancy of their carrier status. They were then treated with successive courses of this tin preparation‡ according to the schedule suggested by Reitler.[3]§ Pertinent data concerning the carriers utilized in this and the subsequent study are presented in Table I. In Fig. 1 are illustrated the time schedule of therapy and the results of stool examination.

It will be noted that none of the established carriers were cleared even temporarily of typhoid bacilli. Carrier 3, who appears to be an exception, evidently recovered spontaneously before treatment was started. Carriers 5, 9, and 16 demonstrated intermittency in their carrier condition, but this characteristic

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*Willard and Harlem Valley State Hospitals.

†All bacteriologic examinations in these studies were performed at the Division of Laboratories and Research, New York State Department of Health.

‡The drug used was kindly furnished by the firm Chemica Ltd., Haifa, Palestine, which markets the drug under the trade name Aldestan.

§Each course consisted of the daily administration by mouth of ten tablets (0.02 Gm. of active ingredient per tablet) for ten days followed by a rest period of one week.

TABLE I. TYPHOID CARRIERS INCLUDED IN STUDY; DATA RELATING TO DURATION OF THE CONDITION

CARRIER	AGE (YR.)	SEX	HISTORY OF TYPHOID FEVER YEARS PRIOR TO STUDY	BACTERIOLOGICALLY PROVED DURATION OF CARRIER STATE (YR.)	LENGTH OF RESIDENCE IN MENTAL HOSPITAL (YR.)	VI AGGLUTINATION TITER AT TIME OF STUDY	BACTERIOPHAGE TYPE	GALL BLADDER X-RAYS
1	69	F	Unknown	6	40	1:10	–	Poor concentration
2	65	F	Unknown	5	26	1:10	–	–
3	55	M	5	5	14	1:40	–	–
4	59	F	Unknown	5	24	1:10	F	Poor concentration
5	77	F	Unknown	8	29	1:20	Could not determine	–
6*	48	F	Unknown	7	20	1:20	F	Stones
7	67	M	Unknown	5	5	1:20	C	–
8*	67	F	Unknown	8	27	1:10	Could not determine	Calcified cyst Poor concentration
9	71	F	Unknown	4	47	1:10	–	–
10	74	F	Unknown	6	25	1:40	–	–
11*	66	F	Unknown	3	19	1:40	E	–
12*	49	F	8	8	13	1:10	Imp. V.	–
13*	50	F	Unknown	3	13	1:10	A	–
14*	50	F	8	8	16	–	Imp. V.	–
15*	58	F	30	9	11	1:10	B_2	–
16	37	F	Unknown	½†	10	Neg.	–	–
17	66	F	1	1	10	1:20	Imp. V.	–
18	74	F	Unknown	½†	17	Neg.	W Form	–
19	83	F	Unknown	7†	12	Neg.	A	–
20	55	F	Unknown	1†	11	Neg.	Imp. V.	–
21*	61	F	Unknown	3	13	1:20	Imp. V.	–

*These carriers also were treated with penicillin and sulfathiazole in a subsequent study described below.

†These individuals were shown to be still carrying *Bacillus typhosus* two and one-half years after the study.

existed before treatment was started. Routine blood and urine examinations were carried out periodically on these carriers throughout the course of treatment in order to determine whether the drug was producing any untoward reactions, but no significant difficulties were encountered.

The value of this procedure in curing typhoid carriers was given a severe test since, for the most part, it could be shown that these individuals were carriers of long duration, even though the mental status of the patients precluded the getting of a history of typhoid fever in all but four instances. Cholecystectomy had not been performed on any of the group. X-ray studies of four of the carriers indicated that either gall stones were present or that the gall bladders functioned poorly. Nevertheless, on the basis on these findings one can hardly attribute any benefit to this therapeutic procedure.

Trial of Penicillin Plus Sulfathiazole in the Treatment of Typhoid Carriers.—The report by Bigger[4] demonstrating the existence of the synergistic effect of sulfathiazole and penicillin on *Eberthella typhosa* in vitro, led to the clinical trial of these drugs by Comerford and co-workers[5] in the treatment of two typhoid carriers. This carefully planned study seemed to demonstrate beyond doubt the cure of these carriers, both through the prompt disappearance of typhoid bacilli from the stools during the course of treatment and the continued

absence of these organisms in daily stool cultures for six months thereafter when the study was terminated. In addition, a striking drop in the typhoid Vi antibody titer of the blood occurred over this period of time. The only reservation one might have concerning the results of this work is that one of the two carriers studied was convalescent from typhoid fever and had carried the organism for only one year, and the other was a chronic carrier who persisted in excreting typhoid bacilli in the stool for ten months following cholecystectomy. Thus neither case was typical of the usual chronic typhoid carrier of long standing with a markedly sclerosed gall bladder containing stones, and it seemed possible that the course of treatment outlined would be ineffective in eradicating the infection in such individuals. Nevertheless, this therapeutic technique appeared to warrant further trial. Since that time an additional paper[6] has reported apparent success in curing a single typhoid carrier with massive doses of penicillin alone.

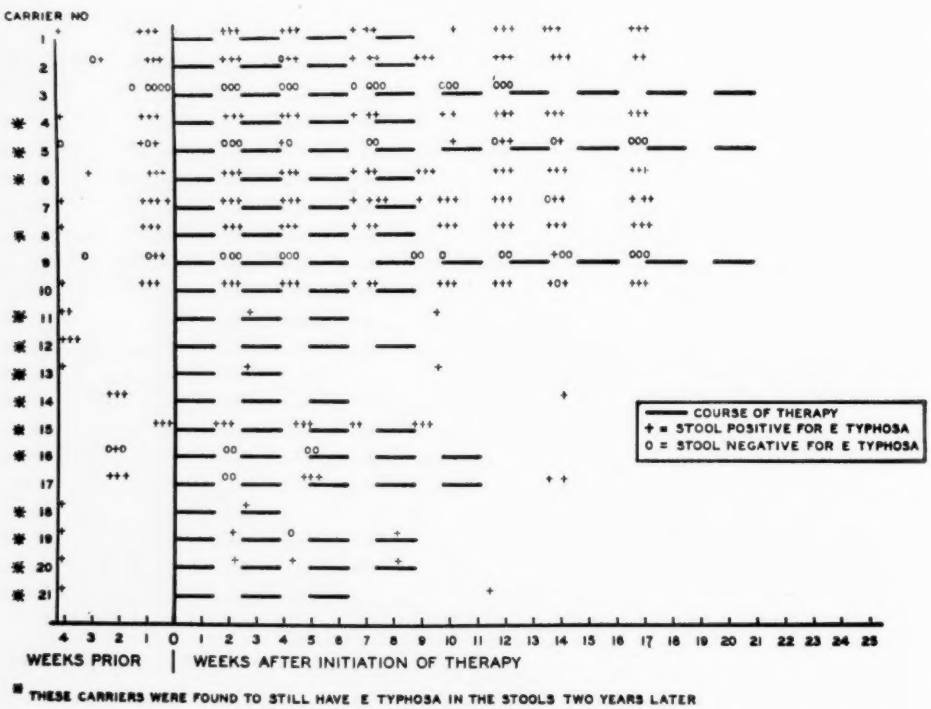
CARRIER NO
COURSE OF THERAPY
+ = STOOL POSITIVE FOR E TYPHOSA
0 = STOOL NEGATIVE FOR E TYPHOSA
WEEKS PRIOR
WEEKS AFTER INITIATION OF THERAPY
* THESE CARRIERS WERE FOUND TO STILL HAVE E TYPHOSA IN THE STOOLS TWO YEARS LATER

Fig. 1.—Treatment of typhoid carriers with tin compound; schedule of therapy and results of stool examinations.

The schedule of therapy utilized by Comerford and co-workers[5] was dictated by in vitro studies which demonstrated the optimal concentrations of sulfathiazole and penicillin needed for inhibition and destruction of the typhoid bacillus. With slight modification, the same schedule was utilized in the present study.

A total of eight chronic carriers, inmates of New York State mental hospitals,* were utilized in this study which was started in August, 1947. (This

*Willard and Harlem Valley State Hospitals.

series represents Carriers 6, 8, 11, 12, 13, 14, 15, and 21 of the first study.) Accumulated records indicated the long-time chronic nature of their carrier state. Daily stool specimens for a period of ten days prior to initiation of treatment confirmed the persistence of the carrier state and the constancy of positive stool findings. None of these individuals were urinary carriers. For an eight-day period sulfathiazole was given by mouth, 1 Gm. every four hours, and crystalline sodium penicillin, in doses of 1,000,000 units, was administered intramuscularly every six hours. Studies of the penicillin blood level* showed uniformly high levels of penicillin one-half hour after administration of the drug which reached a peak of 61 units per milliliter of blood during the fifth day of treatment. Samples collected immediately before successive doses were administered showed at all times more than 0.48 unit of penicillin per milliliter. The results of bacteriologic examination of the stools from these carriers before, during, and subsequent to treatment are illustrated in Fig. 2.

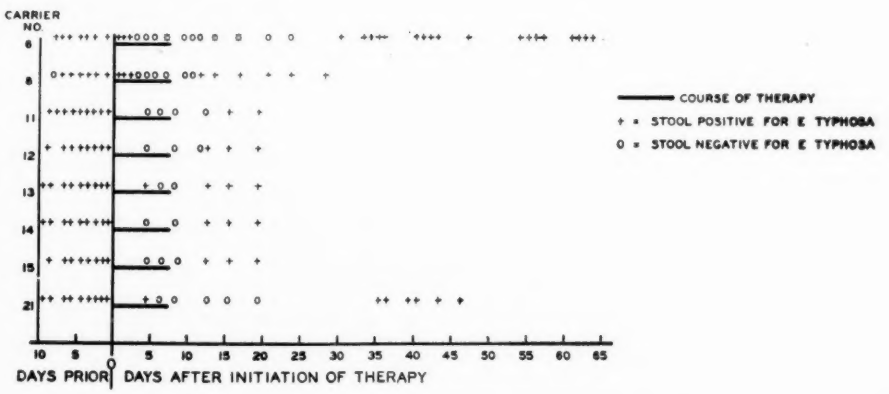

Fig. 2.—Treatment of typhoid carriers with penicillin and sulfathiazole; record of stool examinations and schedule of therapy.

Other pertinent data concerning these carriers already have been presented in Table I. It is evident that in each instance typhoid bacilli disappeared completely from the stool for a period. In two instances careful study failed to reveal their presence for several weeks after completion of the course of treatment. However, the organisms reappeared subsequently and hence in no instance was a carrier actually cured. Several of these individuals had been shown previously to have gall stones, thus accounting perhaps for some of the difficulties in eradicating the infection.

Although none of the carriers treated in this study were permanently cured, it is possible that this therapeutic procedure might have some application in the treatment of the typhoid carrier state which persists occasionally following cholecystectomy or in eradicating the carrier condition early in its development in convalescent cases of typhoid fever. However, it is doubtful that the procedure outlined would be effective in curing the infection in the

*These tests were performed in the laboratory of Dr. George V. Taplin, University of Rochester, Rochester, N. Y.

average chronic carrier, although more prolonged therapy with larger doses of the two drugs might be more fruitful. These findings with respect to the effect of penicillin plus sulfathiazole on *E. typhosa* are in keeping with those reported by Hardy[7] who observed a similar bacteriostatic action on *E. typhosa* with sulfadiazine in nineteen chronic typhoid carriers. It would seem that the only therapeutic measure of proved value for the eradication of the typhoid carrier state is that of cholecystectomy. Experience in New York State[8] indicates that in 68 per cent of chronic carriers undergoing cholecystectomy cure was obtained.

SUMMARY AND CONCLUSIONS

Treatment of twenty-one chronic typhoid carriers with the tin compound heptadekylaldehyde stannoxysterate (Aldestan) failed to demonstrate any effect on the presence of *E. typhosa* in the stool.

The administration of penicillin and sulfathiazole to eight chronic typhoid carriers caused the disappearance of typhoid bacilli from the stool in each individual. In two instances the organisms were absent for at least two weeks following discontinuance of treatment. However, they reappeared in the stool of all the carriers.

The authors would like to acknowledge the cooperation of Dr. Kenneth Keill, Superintendent, Willard State Hospital, and Dr. Alfred M. Stanley, Superintendent, Harlem Valley State Hospital, in making these studies possible.

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THE OCCURRENCE OF SALMONELLA BLEGDAM IN LOUISIANA*

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SALMONELLA blegdam was first isolated in 1929 at State Serum Institute,[1] Copenhagen, Denmark, from the blood of a patient in the Blegdam Hospital[2] suffering from pneumonia of the right lower lobe. This organism was not described, however, until 1935 by Kauffmann.[3] In 1941, Fournier[1] again found *S. blegdam* in the blood of a patient in Shanghai, China. Atkinson and associates[4] recorded in 1944 the occurrence of this bacterium in blood and feces of four cases of enteric-like fever in soldiers in New Guinea. During the first year of the United States reoccupation of the Philippines, Stevens[2] isolated four strains of *S. blegdam* and noted his findings in 1946. Two of these four isolations were from the blood of American infantry soldiers with symptoms of paratyphoid infections; one was from the feces of a soldier without apparent enteric fever, and the other one was from an ulcerative lesion on the ankle of a Filipino patient with no gastrointestinal symptoms. Cobley and Wilson[5] in 1946 reported a case of *S. blegdam* septicemia and suppurative pericarditis with recovery in an Australian soldier. This Salmonella was isolated both from the blood and pericardial fluid and identified by Atkinson and co-workers. Fenner and Jackson[6] described in 1946 fifty cases of enteric fever due to *S. blegdam* again in Australian soldiers from New Guinea. The diagnosis was established in seventeen cases by the identification of the organism by Atkinson and associates and in the remainder of the cases by clinical, epidemiologic, and serologic findings. In 1947, Atkinson and associates[7] recorded additional *S. blegdam* isolations from blood and feces of soldiers and natives in New Guinea and Bougainville Island, but as well as can be ascertained seventeen of these strains were obtained from the cases reported by Fenner and Jackson. Five strains from mice and one strain from a guinea pig in a laboratory stock suffering from an epidemic also were found by Atkinson and co-workers.[7]

We wish to report at this time the isolation§ in December, 1947, of a strain of *S. blegdam* from the feces of a patient in the Southern Baptist Hospital presenting an enteric type of fever and an extensive erythema. During January, 1948, an isolation of *S. blegdam* also was made§ from the blood of a patient in the Touro Infirmary with upper respiratory and gastrointestinal symptoms thought at first to be a viral infection. It is of clinical interest that repeated blood counts during the period of hospitalization showed a persistent leucopenia with depression of the granulocytic series.

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§We are grateful to Dr. Erich Seligman and Dr. Ivan Saphra, National Salmonella Center, New York, N. Y., for the final identification of both strains of *S. blegdam* isolated by us. Acknowledgment is also made to Miss Louise Cargile, Southern Baptist Hospital, and to Miss Madeleine Page, Touro Infirmary, for their technical assistance.

TABLE I. DATA PERTINENT TO INTERPRETATION OF BACTERIOLOGIC RESULTS

	SOUTHERN BAPTIST HOSPITAL					TOURO INFIRMARY				
Type of illness	Enteric type of fever; extensive erythema					Upper respiratory and gastrointestinal symptoms; fever				
Source of isolation	Feces, 11 days after hospitalization					Blood, 7 days after hospitalization				
Other specimens examined	Two blood cultures (second week); results: negative for Salmonella					Stool cultures (second and third weeks) and urine culture (second week); results: negative for Salmonella				
Agglutination tests		FEBRILE HOSPITAL DAY					FEBRILE HOSPITAL DAY		DAYS AFTER DISCHARGE	
		3	9	12	20		5	11	9	69
	Ty. O	0	0	0	0	Ty. O	Pos. 1-40	Pos. 1-40	Pos. 1-160	Pos. 1-320
	Ty. H	0	0	0	0	Ty. H	Pos. 1-160	Pos. 1-160	Pos. 1-320	Pos. 1-320
	Para A	0	0	0	0	Para A	Pos. 1-80	0	0	Pos. 1-160
	Para B	0	0	0	0	Para B	Pos. 1-40	0	0	Pos. 1-160
	Br.	0	0	0	0	Br.	0	0	0	0
	Pr. X19	0	0	0	0	Pr. X19	0	0	0	0
	N.B.: Serum collected 7 weeks after hospitalization showed widal pos. 1-800 according to results obtained at National Salmonella Center					TUBE-LIVING ANTIGENS				
						S. blegdam	X	X	Pos. 1-1280	Pos. 1-320
						S. typhosa	X	X	X	Pos. 1-640
						S. typhi murium	X	X	X	Pos. 1-160
						S. oranienburg	X	X	X	0
						S. virginia	X	X	X	Pos. 1-160
						S. javiana	X	X	X	Pos. 1-320
						S. newington	X	X	X	Pos. 1-160
						Proteus X19	X	X	X	0
Typhoid-paratyphoid vaccination	No record of previous typhoid vaccination; negative agglutination tests indicate no vaccination					1943, 3 injections; Apr., 1945, 3 injections; Apr., 1946, booster; Feb., 1947, booster				
Duration	Discharged 41 days after admission to hospital					Discharged 16 days after admission to hospital				
Epidemiology	Patient's husband was a member of Armed Services stationed in various parts of South Pacific Theatre during World War II; two stool cultures on husband were negative for *S. blegdam;* patient had eaten sea food prior to onset of symptoms					Patient (student nurse) visited home 1 to 2 weeks prior to onset where brother and mother had been ill; cases diagnosed "flu"; diarrhea, nausea, vomiting, but no fever; duration 3 to 4 days; brother served in U. S. Army 1944-45 in New Guinea, New Georgian Islands, New Caledonia, etc.; no laboratory studies available on either mother or brother				

We feel that these findings are worthy of reporting since we have been unable to find any reference in the literature to the previous isolations of *S. blegdam* in the United States. In correspondence on December 24, 1947, with Seligman,* we were informed that this species of Salmonella had never before been found in this country. Of further interest is that the second case from which this rare species was isolated again in New Orleans simulated clinically the original one described by Kauffmann[3] in Copenhagen. These two strains of *S. blegdam* are thought to be the first isolated from anyone in the United States and from individuals who had never left this country.

Biochemical Activities.—The isolation of *S. blegdam* from both blood and feces followed the usual procedure in the study of enteric pathogens. In Kligler's agar, the production of acid and gas in butt, alkaline reaction on slant and hydrogen sulfide occurred. It failed to ferment lactose, sucrose, salicin, dulcite, and inositol. Fermentation with gas production occurred in glucose, mannite, maltose, xylose, sorbitol, arabinose, and rhamose. Indole was not formed. Urea was not split. Milk showed no coagulation. Gelatin was not liquefied.

Antigenic Structure.—According to Bergey's Manual, *S. blegdam* possesses the formula: IX, XII: g,m,q: —. Because this organism possesses the somatic antigen IX, it is classified in the serological Group D. It is a monophasic flagellated bacillus existing only in phase 1. In our laboratories we were able to identify the somatic antigens only. The identification of the flagellar antigens was made by the National Salmonella Center. (See Table I.)

SUMMARY

S. blegdam was isolated for the first time in Louisiana and probably in the United States by Newton from the feces of a patient suffering with enteric type of fever and extensive erythema during the fifth week after onset.

The second isolation of *S. blegdam* in Louisiana was by Holt from the blood of a patient with upper respiratory and gastrointestinal symptoms closely resembling clinically those of the patient from which *S. blegdam* was first isolated by Kauffmann as well as the type of Salmonella fever described by Fenner and Jackson in which complications developed. The organism was found during the second week of the infection in a student nurse.

Neither patient had traveled outside the United States. Both patients gave histories of contact with members of the Armed Forces who had previously served in New Guinea and various posts in the South Pacific Theatre during World War II. No epidemiologic facts, however, could be established on the carrier state of these two contacts or on other possible sources of infection.

Serologically, results obtained on routine febrile and in vitro agglutination tests by Holt failed to be of value in the diagnosis of this infection and led to confusion. Attention is called to the close antigenic relationship of *S. blegdam* to *S. typhosa*.

The final identification of this organism was by analysis of the antigenic structure of which it is composed.

Routine vaccination against typhoid-paratyphoid infections as used in this country afforded no protection against *S. blegdam* in one of the patients.

From a review of the available literature we could find recorded the isolation of only thirty-one strains of *S. blegdam* from human sources prior to the additional two strains now being reported by us.

Diagnosis of this Salmonella infection is best established by the isolation of *S. blegdam* from blood, stool, urine, or any source involved.

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EXPERIMENTAL THERAPY OF GENERALIZED TORULOSIS IN RATS WITH STREPTOMYCIN

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IT HAS been pointed out previously in an excellent monograph by Cox and Tolhurst[1] that if any treatment favorably influences the course of torulosis it is as yet unknown. However, a localized lesion may heal spontaneously.

Many forms of therapy have been used to little or no avail. The acriflavine dyes[2, 3] have been administered by several routes, including the intrathecal route, with eventually fatal results. Beck and Voyles,[4, 5] Hamilton and Tyler,[2] Marshall and Teed,[6] and many others have used the sulfonamides and potassium iodide with few and very uncertain beneficial results. Jones and Klinck[7] in therapeutic experiments in mice demonstrated that sulfadiazine and penicillin were of no value. The subjects of clinical cases reported by Harford and co-workers[8] and Cox and Tolhurst[1] did not benefit from penicillin. Stoddard and Cutler[9] and Hoff[10] have amply demonstrated the ineffectiveness of the injection of the killer organisms and the other Torula antigens. Mezey and Fowler[11] report one case with a short follow-up in which some benefit was derived from the use of intravenous 5 per cent alcohol and dextrose solution. Shapiro and Neal[12] used colloidal silver and immune rabbit serum intraspinally without favorable result. X-ray therapy has been tried without benefit.[13]

Since little or no success with any of the afore-mentioned therapeutic agents has been achieved, a therapeutic test using streptomycin has been carried out in rats.

In vitro titrations of streptomycin against Torula* showed little anticryptococcic activity. Apparently there was no inhibition of the same strain used in this experiment. However, Whiffen and co-workers[14] discovered a highly effective antibiotic against Torula in the liquors in which streptomycin was prepared. This agent was effective in vitro but was extremely toxic in vivo and therefore could not be used in animal experiments.

Forty-seven medium sized albino rats were selected for this trial since it had been shown previously that the rat is easily infected with Torula through intraperitoneal inoculation. In addition, it does not succumb to the infection too readily. An inoculum was prepared with the same strain in the same fashion described by Beck and Voyles[5] in 1946. Each rat received 100,000 organisms intraperitoneally.

One week after the inoculation with the standardized Torula suspension treatment was begun. The rats were separated into two groups, the control group numbering twenty-one and the treated group numbering twenty-six. Both groups were maintained under identical conditions and were fed the same type food. In no way was a differentiation made except in that one group was treated with 3,000 units of streptomycin in three doses every twenty-four hours for twenty-one days. The drug was given subcutaneously over the abdomen.

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No attempt was made to check the levels in the blood. At the end of the three-week period, two of the control group and one of the treated group had died. In ten weeks the experiment was terminated, and at this time there were 61.6 per cent (sixteen) of the treated animals alive and 33.3 per cent (seven) of the untreated controls. In addition it should be pointed out that in ten of the animals in the treated group no lesions could be found either grossly or microscopically at post-mortem examination. Lesions were found in all of the control animals (untreated) with the exception of one animal which died an accidental death by drowning soon after the experiment began.

The clinical appearance of the rats which were being treated showed a remarkable difference from those in the control group. The latter group developed lassitude and somnolence and appeared chronically ill; whereas the treated group showed few or no signs of illness.

In summary, forty-seven animals (rats) were inoculated with *Torula histolytica;* twenty-one served as a control group and twenty-six served as a treated group. Treatment consisted of 3,000 units of streptomycin given subcutaneously in normal saline in divided doses daily. Sixty-one per cent of the treated animals and thirty-three per cent of the untreated animals survived. In ten of the treated group no lesions could be found at post-mortem examination. In only one of the untreated did we fail to find the lesions.

CONCLUSION

In this experiment streptomycin seems to have exerted a beneficial effect upon experimental torulosis. It is not known whether its effect is due to the streptomycin or to some additional factor present in its market preparation.

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NUTRITION AND EXPERIMENTAL DIABETES

I. The Diabetic Response of Weanling Rats to Intravenous Doses of Alloxan

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THE induction of diabetes in small animals by subcutaneous or intraperitoneal injection of solutions of alloxan monohydrate is an uncertain procedure. The wide variations in response with these routes of administration are in part related to the rapid inactivation of alloxan by body fluids. In the course of work involving the use of large numbers of young diabetic rats, we found it necesssary to investigate the relationship of the intravenous dose of alloxan to (1) the selective destruction of pancreatic cells which will lead to diabetes without concomitant injury to other visceral organs, notably the liver and kidneys (the validity of metabolic studies in animals made diabetic with alloxan is dependent upon this selective anatomical alteration), (2) the incidence of diabetic response among injected animals, and (3) the extent and permanency of the defect of carbohydrate metabolism produced.

EXPERIMENTAL

Young albino rats of the Hisaw or Sherman strains were used. We could detect no strain difference in response to alloxan. The animals were maintained on dog food* before injection. All animals ranged between 30 and 50 grams in weight at the time of injection. A 3 per cent solution of alloxan monohydrate† was prepared in sterile distilled water immediately before each injection. It was found that the available preparations of alloxan monohydrate are often variable in appearance and solubility. The preparations used therefore were assayed chemically by the manometric procedure of Archibald[1] and the dosage was adjusted to the amount of alloxan in the sample. The solution was drawn into a 0.5 ml. tuberculin type of syringe equipped with a one-half inch No. 26 needle. The animal was placed in a mailing carton with the tail protruding through a hole in the center of a cork stopper used to close the carton. The container was conveniently clamped to a ring stand. Cleaning the tail surface with a soapy cloth followed by a brisk rub with a dry cloth will adequately dilate the four tail veins without heating. A tourniquet is not necessary.

The needle is introduced, bevel up, directly over the vein and on the distal one-third of the tail. Veins over the proximal third, although apparently larger, are injected with difficulty. The vein is closely attached to the skin,

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*Gaines Dog Meal, Formula A-13H.

†Eastman Kodak Company, Rochester, N. Y.

so the needle must be inserted at a small angle with the tail axis. When blood appeared at the syringe nipple, the calcualted volume of alloxan solution was injected quickly. When undue resistance or perivascular blanching occurred, indicating extravasation, the animal was discarded. With this procedure one can soon learn to inject about twenty-five rats per hour.

The animals were placed in group cages and at intervals were placed in individual metabolism cages for twenty-four hours. In young rats several criteria may be used for determining the presence of diabetes. The data below are based upon twenty-four-hour urine glucose excretion as measured by a photometric modification of the method of Somogyi[2] and blood sugar determinations by the method of Reinecke[3] after a preliminary five-hour fast. Failure to gain weight, polydipsia, polyuria, and, after several weeks, cataract formation are also manifestations of diabetes. A fasting blood sugar above 150 mg. per cent or twenty-four-hour urine glucose excretion of more than 0.1 Gm. was considered an indication of diabetes.

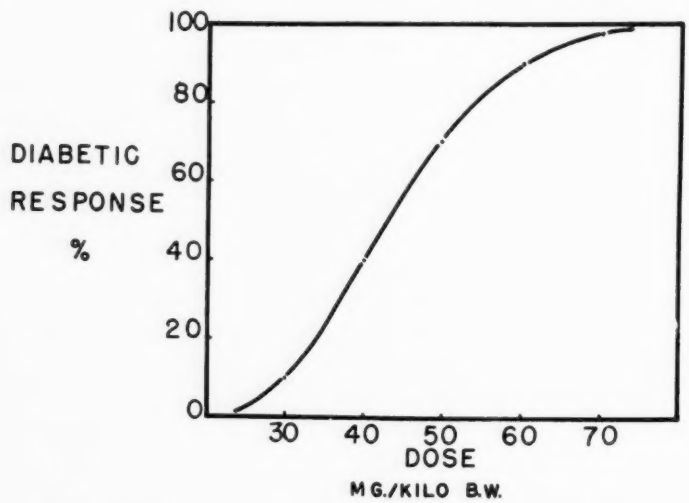

Fig. 1.—The forty-eight hour diabetic response of weanling rats to the intravenous injection of alloxan monohydrate.

Fig. 1 represents the typical forty-eight-hour response of several groups of animals to the various doses of alloxan used. The incidence of diabetes thereafter is to some extent determined by the nature of the diet fed. In general the dog food diet was found to give the best survival. When a purified diet containing 18 per cent protein was used, 100 per cent mortality was obtained in ten weeks contrasting with 100 per cent survival in a similar group of animals maintained on the dog food diet. Table I illustrates the effect of certain diet modifications upon the course of the diabetes, with mortality as a criterion of dietary adequacy.

The groups of rats in Table I were composed of animals with comparable severity of diabetes as judged by the criteria listed. They were placed upon the various diets five days after the injection of alloxan. It will be noted that al-

TABLE I. THE EFFECT OF DIET UPON SURVIVAL IN YOUNG DIABETIC RATS (ALLOXAN)

DIET	NUMBER OF RATS	SURVIVORS DAYS 14	28	56	70	TOTAL MORTALITY (%)
Dog food*	8	8	8	8	8	0
10% Protein	7	5	3	2	2	72
20% Protein	8	8	7	4	2	75
40% Protein	8	7	6	4	2	75
40% Protein plus 1% NaCl in water	7	6	5	4	2	72
18% Protein purified	20	14	11	6	0	100

*Gaines Dog Meal, A-13H.

though the mortality at seventy days was approximately the same with various levels of protein, the early mortality was higher with the lower protein levels. The survivors at seventy days represent the animals with mild diabetes. In the various diets the protein was increased at the expense of carbohydrate. The inadequacy of the purified diet, as judged by mortality, is striking when compared with either the dog food diet or the semipurified diets (containing 10 per cent brewers' yeast as a source of B vitamins). Addition of 1 per cent sodium chloride did not significantly alter the survival.

The influence of diet upon the severity of diabetes and the survival of the diabetic animals will be the subject of a later publication.

DISCUSSION

The immediate mortality (about 7 per cent) in animals receiving intravenously 70 mg. of alloxan per kilogram of body weight has been caused by injury of the liver and kidneys of these animals. The susceptibility of animals to this extrapancreatic toxicity of alloxan appears to be as variable as diabetic susceptibility. Doses above 70 mg. per kilogram lead to increasing incidence of this complication.

Selection of the optimum dose is thus determined by two perimeters. First, doses below 40 mg. per kilogram give diabetic responses in so few animals that many animals and much time are lost. Second, doses above 70 mg. per kilogram often lead to injury of organs other than the pancreas, thus complicating later metabolic studies.

The 60 mg. per kilogram dose level has been found most practical. The 10 to 20 per cent of nonresponding animals can be used as alloxan controls in many experiments. Such controls may in part answer the criticism that alloxan may act as a nonspecific cellular toxin leading to complications other than diabetes. Thus in our work it has been useful to control each experiment with animals not injected with alloxan and with alloxan-injected, nondiabetic animals. Although we have not studied these latter control animals with glucose tolerance tests, the twenty-four urine glucose excretion is in effect a tolerance test and would be expected to reveal animals with minimal diabetes.

Histologic studies of animals receiving alloxan have revealed no evidence of renal or hepatic damage attributable to the drug in doses of 60 mg. per kilogram of body weight or less. Animals receiving higher doses and dying within forty-eight hours frequently have shown such lesions. Of thirty animals receiving 70

mg. per kilogram which survived the injection forty-eight hours, only one animal later showed cirrhotic changes in the liver with ascites, and this after a period of fourteen months. This was believed to be a late result of mild hepatic injury by alloxan.

Consideration of the severity of diabetes among the responding animals indicates that with increasing doses the number with severe diabetes increases. This would seem to indicate a range of sensitivity among animals extending from the few animals which respond with severe diabetes through the majority which respond moderately to the few who will not respond at all. Experience with repeated injections of those animals that do not respond indicates that they often are resistant even to 80 to 100 mg. per kilogram of alloxan.

It should be emphasized that young rats made diabetic with alloxan offer an extremely sensitive experimental tool for study of nutritional relationships in the course of diabetes. Measurements of body weight, water consumption, or urine excretion offer a simple means of assaying the influence of experimental procedures.

SUMMARY

The diabetic response of weanling rats to intravenous doses of alloxan has been studied.

A procedure has been described which will allow production of alloxan diabetes in the majority of injected animals with minimal occurrence of extrapancreatic alloxan injury.

An experimental plan has been suggested which will allow more adequate control of experiments by utilization of nonresponding alloxan-injected animals as additional control animals.

The course of animals made diabetic with alloxan and surviving more than forty-eight hours is determined in part by diet. Survival is greatest in animals fed a diet of dog food and poorest in animals on a highly purified diet. Higher levels of protein and decreased amounts of carbohydrate favored survival on the purified diets.

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MYELITIS FOLLOWING THE ADMINISTRATION OF NEOARSPHENAMINE

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ONE of the hazards involved in the therapeutic use of arsenicals is the danger of central nervous system reactions. These accidents although rare are serious, causing approximately one-half the deaths from arsenical therapy.[1] In a survey of 496,253 protocols, one death due to central nervous system reaction occurred for every 5,398 persons treated and every 28,768 injections given. In a group of 158 patients with central nervous system reactions, the mortality rate was found to be 76 per cent.

These reactions include encephalitis, encephalomyelitis, and myelitis. Encephalitis is by far the most common. It has been widely described and the usual picture of headache, fever, and vomiting followed by convulsions and coma is well known. Encephalomyelitis, a more rare complication, is less widely described. The signs of cerebral involvement usually precede and may mask the evidences of cord lesions. About half these patients have been considered to have encephalitis alone until post-mortem examination showed unsuspected pathologic changes in the spinal cord. Because myelitis is uncommonly reported and infrequently considered, the following protocol is presented.

A 25-year-old woman was given two intravenous injections of neoarsphenamine by the family physician because she suspected she had been exposed to syphilis. A man with whom the patient had been in intimate contact had been discovered recently to have a secondary syphilitic rash. The patient had no signs of syphilis and a serologic test for syphilis was negative. Nevertheless it was decided that she should be treated. Accordingly, the patient was given 0.3 Gm. of neoarsphenamine intravenously without untoward reaction. Seven days later she was given 0.45 Gm. intravenously.

Two hours after the second injection the patient had a severe shaking chill followed by a fever of 39.5° C. and moderately severe malaise. She went to bed and for two days had recurrent chills and fever and anorexia. On the first day the physician gave her 600,000 units of procaine penicillin. On the morning of the second day a faint, pink, nonpruritic rash of small irregular macules appeared on the face, arms, and neck. The patient also noted that her throat was slightly sore and that her eyes burned. The evening of the second day following the second injection of neoarsphenamine the rash faded and the chills and fever subsided, but the malaise increased. The patient noticed then, for the first time, that her feet felt numb and that she had difficulty in walking. She was barely able to walk with assistance to the bathroom and void.

In the twelve hours that followed, a painless sensation of numbness rose up the patient's legs to the trunk, arms, and neck. As this occurred, all voluntary motion was lost in the legs which lay limply on the bed. The patient also developed urinary retention. She was mentally clear and had no pain. There was no difficulty in talking, swallowing, or breathing and no difficulty with vision. When the sensory defect was at its maximum the patient noted transient impairment of voluntary motion in the arms and hands. The sensation of numbness

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receded from the neck, arms, and upper chest as rapidly as it had appeared; hence on the following morning when she was seen by the physician, a sensory level was apparent above the navel. At this time (the third day following the last injection of neoarsphenamine) there was a low-grade fever, malaise was gone, and appetite was returning. Complaints were: sore throat, loss of sensation in trunk and legs, inability to move the legs, and urinary retention.

The patient remained at home in bed three more days and did not consider herself seriously ill although she had a slight fever of about 38° C. and was bothered by her sore throat. The sensory level did not change nor did motor function in the legs improve. The patient could sit without support after being helped to a sitting position. She voided spontaneously, slowly, at long intervals (sixteen to twenty hours), passing large amounts of urine. On the sixth day frequency and urgency developed quite suddenly, with voiding every twenty to thirty minutes. Distressed by this and discouraged by the lack of improvement in the function of her legs she came to the New York Hospital.

On admission the patient appeared moderately ill but was alert, cooperative, and oriented. Temperature was 37.5° C. There were minimal but generalized lymph node enlargement, mild conjunctivitis, and pharyngitis. A single, small, faintly pink, irregular macule was present on the left cheek. The remainder of the general physical examination was not contributory. Examination of motor and sensory functions showed no abnormalities of the head or neck. There was no weakness or wasting in the arms. Rapid, rhythmic, alternating motion was slightly impaired in the right arm, and the biceps, supinator, and pronator reflexes were slightly more active than on the left. Sensory perception was normal in the neck, arms, and trunk above the level of D-8. From D-8 to D-10 there was dysesthesia to pin prick, light touch, and temperature testing. Below D-10 there was anesthesia to these modalities but vibration and position sense were intact above and below this level. There was no sweat level or pain on percussion over the spine. Superficial abdominal reflexes were absent. The patient could sit without support if assisted to the upright position. The legs were symmetric and without wasting, but were completely flaccid. The only voluntary motion possible in the legs was a slight degree of flexion in the right knee. The tendon reflexes were normal but slightly more brisk in the right leg. Plantar responses were extensor. There were mass withdrawal responses in the legs. Rectal sphincter tone was normal but the perianal region was anesthetic. The patient was able to void spontaneously but was forced to urinate every twenty to thirty minutes in order to escape incontinence of urine.

On admission, urine analysis showed only an occasional white cell. Repeated analysis showed no albumin or red cells. The blood urea nitrogen was 11 mg. per 100 milliliters. The Mazzini reaction was negative and remained so. Blood counts were within normal limits. Heterophile agglutinations were negative on admission and ten days thereafter. Cultures from the throat showed a mixed flora with no predominating organism. A patch test with neoarsphenamine on the seventh hospital day produced a 2 cm. indurated erythematous area in sixty hours. Lumbar puncture on admission gave crystal clear fluid with no evidence of block or increased pressure. Repeated cerebral spinal fluid cultures and Wassermann reactions were negative. Cerebral spinal fluid sugar and chloride determinations were within normal limits. The results of repeated lumbar punctures were as follows.

DAY	LYMPHOCYTES	POLYMORPHONUCLEARS	PROTEIN
1	3	0	55
2	4	1	39
7	6	2	24
13	5	0	30

There was slow but steady improvement which began with a slight regression of the level of anesthesia on the first hospital day. On the second hospital day the patient disclosed that she had received two injections of neoarsphenamine. Because of previous reports of myelitis following neoarsphenamine injections, it was decided to begin British anti lewisite (BAL) therapy. A total of 1,300 mg. of BAL was given over a four-day period. By the eighteenth hospital day anesthesia was confined to a few spotty areas on the lower legs and in the perianal region. These changes persisted and in the entire area which was

formerly anesthetic there was dysesthesia to pin, light touch, and temperature testing similar to that found in the area from D-8 to D-10 on admission. The patient gradually regained voluntary motion and strength in the legs, but improvement was retarded by the transient appearance of a moderate degree of extensor spasm. The reflex abnormalities in the arms and legs did not change with the exception of the plantar responses which became flexor. The patient was able to walk without support on the thirty-seventh hospital day. She continued to have frequency of urination but the interval had increased to one to two hours.

Most standard texts do not list arsenical myelitis as one of the toxic manifestations of arsphenamine therapy. So few cases have been reported that it is impossible to draw inferences as to cause and effect as has been done with arsenicals and encephalitis. In all, there are only nine cases similar to that of the patient herein described.[3] The chance occurrence of myelitis of unknown cause in a patient receiving arsenicals must be considered. The case reports are so few that no clear-cut clinical picture is typical. However, many of the reports are strikingly similar. Scott and Reinhart[4] describe a patient in whom the onset and progression of symptoms were almost identical and followed the second injection of neoarsphenamine. This patient, however, did not survive. Many patients have been suspected of having had Herxheimer reactions and only one previous patient did not have syphilis. However, in the few that have been autopsied the histopathology of the cord was similar to that in the brains of patients with encephalitis and in the cords and brains of those with encephalomyelitis.

In the New York Hospital case the possibility of a Herxheimer reaction may be waived. The possibility of intercurrent myelitis of other cause remains and cannot be eliminated.

BAL has been shown by Stocken and co-workers to protect rats from lethal doses of therapeutic arsenical compounds.[5] Eagle and Magnuson[6] have reported fifty-five cases of arsenical encephalitis treated with BAL. The over-all mortality rate was 11 per cent as opposed to 76 per cent of an earlier untreated series. Severe cases with convulsions, coma, or both were observed to respond dramatically in twenty-four to seventy-two hours, particularly if BAL was given early. There is now a convincing body of evidence that the toxic effects of arsenicals are due primarily to the fact that they combine with and block physiologic enzyme systems vital to the cellular economy. The antidotal action of BAL is referrable to its ability to remove arsenicals from combination with these enzyme systems in the cells.[7] There has been discussion as to whether the central nervous system reactions from arsenicals, particularly myelitis, are due to toxicity or sensitivity. The fact that BAL has been dramatically effective in encephalitis suggests that the mechanism here involves toxicity, reversible with early BAL therapy. Since the histopathology is similar in encephalitis and myelitis, it can be postulated that myelitis is a toxic reaction which should respond just as dramatically.

In the New York Hospital case, BAL was given too late to allow inferences concerning therapeutic or diagnostic values. Yet the outcome here is unusual since only two of the previously reported patients survived, one with severe residuals. If myelitis following the injection of neoarsphenamine is due to toxic effects of this agent it should respond to BAL when given early. It would be one variety of myelitis for which there is a specific therapeutic agent.

SUMMARY

Myelitis following the administration of neoarsphenamine is a rare but serious complication of arsenical therapy. It is suggested that, if arsenical compounds have been given to a patient developing myelitis, BAL therapy be instituted.

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LABORATORY METHODS

RECOVERY AND ESTIMATION OF RADIOACTIVE ISOTOPES FROM BIOLOGIC TISSUES

I. Gold

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IN ORDER to study the distribution of gold in animal and human tissues, it was necessary to develop a rapid and quantitative method for the recovery and estimation of this element. Toward this end, use was made of the experience gained, during 1942 and later, with the electrodeposition methods employed on the Manhattan Project (unpublished papers[1, 2]). Investigations along these lines also were encouraged by the successful use of electrodeposition in the determination of radioactive iron by Ross and Chapin,[3] Hahn,[4] and others.

By the use of the electrodeposition technique, thin, uniform films of the radioelement may be obtained which then can be used for the determination of radioactivity. Standard Geiger or alpha counters are employed for these measurements, as the case may be.

As early as 1918, DeWitt, Caldwell, and Leavell[5] had shown that it was possible to determine gravimetrically the amounts of gold present in biologic tissues after electrodeposition on platinum electrodes. Details of the method employed by these authors could not be found, although a brief description was furnished: namely, Kjeldahl digestion, followed by evaporation to about 1 c.c.; treatment with aqua regia and ammonia, and neutralization with HCl. The solution was filtered, made slightly acid with HCl, and buffered with phosphoric acid-sodium phosphate. Electrodeposition was carried out in 40 c.c. of solution at 60° C. and 1 to 1.2 volts. Current density was not given. Recoveries of 80 to 100 per cent were obtained with 0.02 to 0.3 mg. of gold.

Scott[6]† gives methods for the determination of gold in electroplate baths and also outlines a microanalytical procedure,‡ all using platinum anodes and cathodes and potassium cyanide plating solutions. Procedures for the commercial plating of gold are given in standard references and trade journals.[7]§[-9] Most of these methods employ gold anodes and either chloride or cyanide plating baths.

In general, the method herein described follows closely the standard procedures for the separation and electrodeposition of gold.

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†1:435.

‡2:2518.[6]

§Pages 199-210.[7]

EXPERIMENTAL

Electrodeposition Apparatus.—The anode consists of 0.040 inch diameter platinum wire wound in a flat spiral. The vertical length of wire is sealed into glass and both are connected into a suitably drilled brass rod 3/8 inch in diameter. The latter is fixed in the chuck of a stirrer rotating at about 300 revolutions per minute.

Cathodes are 1½ inch diameter disks, 0.002 inch thick, either of platinum or gold-plated copper. Platinum must be used for exacting work, but for reasons of economy the plated copper was substituted in carrying out these experiments.

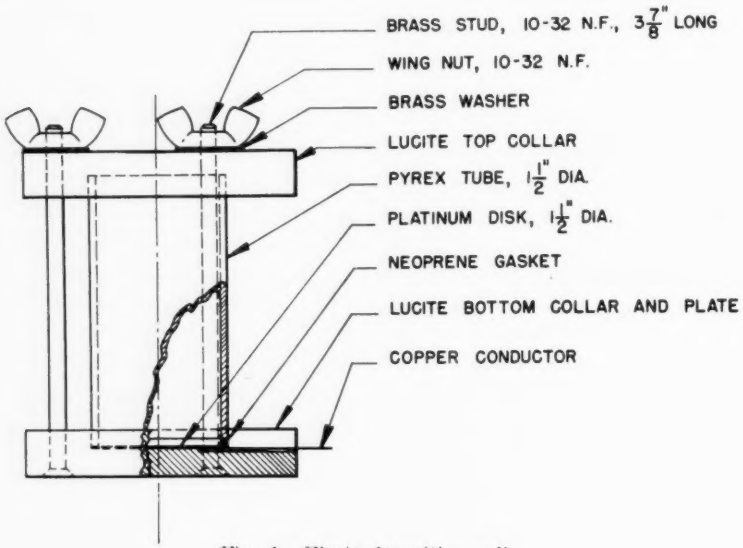

Fig. 1.—Electrodeposition cell.

The electrodeposition cell* shown in Fig. 1 uses a 1½ inch outside diameter Pyrex tube, 3 inches long.

Operating current is supplied from two cells of a 6-volt storage battery. An ammeter with a range of 0 to 1 ampere and a 1-watt, 20-ohm rheostat are placed in series with the electrodeposition cell. The actual apparatus† consists of twenty-four such units arranged in two banks of twelve each. Each unit has a toggle switch for throwing the ammeter in or out of the circuit. For work with gold, a better arrangement would be to use an ammeter with a scale of 0 to 0.2 amp. and a variable resistance of 50 ohms. Each unit should have a fuse to protect both the ammeter and the resistance, and a voltmeter should be provided to measure the potential drop between anode and cathode. The apparatus used for this work had been built for another purpose and could not be modified in the time available.

Procedure.—The weighed tissues are digested in 25 ml. Erlenmeyer flasks, using either aqua regia or concentrated nitric acid plus superoxol. In either

*Detailed drawings can be furnished upon request.

†Designed and constructed by Mr. Robert Loevinger of this laboratory.

case, only small amounts of reagent are added at one time. If excessive frothing is encountered, 1 drop of octyl alcohol is added. Complete oxidation of the latter, as well as the tissue, must be accomplished before proceeding further.

As a carrier, 2.0 ml. of a reagent grade gold chloride solution may be added either before, during, or after digestion, although addition at an early stage in the digestion is recommended. The concentration of this solution should be accurately known, and the solution should contain approximately 5 mg. per milliliter of metallic gold as the chloride. The concentration of gold in the carrier solution is determined either by electrodeposition on tared platinum disks or by evaporating a known volume to dryness in a tared porcelain crucible, followed by heating to decompose the chloride, cooling, and weighing.

Upon completion of digestion, excess nitric acid is removed by adding successive small amounts of concentrated HCl with heating. The resulting solution is evaporated to 1 to 2 ml. and transferred to a 15 ml. centrifuge cone. Only a roughly quantitative transfer is needed since the electrodeposited film is to be weighed, and recoveries as low as 90 per cent or even 80 per cent are acceptable.

The separation of the gold from all or nearly all of the associated elements in the tissue digest is accomplished by reducing the gold to the metallic state. The reducing solution is made up to contain approximately 10 per cent $NH_2OH{\cdot}HCl$ and 0.4 per cent $FeSO_4{\cdot}7H_2O$. Two milliliters of a freshly prepared solution are used for each 10 mg., or less, of metallic gold. Reduction is hastened by placing the cones in a beaker of hot water which is kept just below the boiling point for fifteen to thirty minutes. The ferrous sulfate is omitted from the reducing solution when urine and feces samples are to be run.

After centrifugation, the supernatant liquid is discarded. The centrifuge cone wall and the gold may be washed and centrifuged, and the wash solution also discarded. Although this is not considered necessary for routine work, it was carried through when the set of determinations shown in Table I was made.

The pellet of precipitated gold is readily dissolved in a mixture of 4 drops of hydrochloric acid plus 1 drop of nitric acid. Placing the cone in a beaker of hot water accelerates the reaction, which is usually complete within fifteen minutes.

The aqua regia solution of gold is made alkaline with 5 ml. of 1N sodium hydroxide. After mixing, this solution is transferred to the electrodeposition cell. The latter should be assembled with a tared and numbered disk, and should have 2 ml. of a 5 per cent solution of potassium cyanide in 0.1N sodium hydroxide covering the disk before the gold solution is added. The centrifuge cone is washed with distilled water, which is also poured into the cell. In routine work this is accomplished by filling the cone once with distilled water, followed by transfer to the cell.

The anode is placed in the solution at about ¾ to 1 inch from the cathode. The current is adjusted to approximately 85 milliamperes. Since the actual plating area is about 8.5 sq. cm., the current density will be 10 Ma. per square centimeter, or approximately twice the current recommended for commercial electroplating.*

*Page 202.[7]

Deposition is allowed to proceed for one hour at room temperature. At the end of this time an additional 2 ml. of the cyanide solution are added, followed by another hour of electrodeposition.

Upon removal of the anode the cell contents are discarded, and the cell is washed with distilled water. After disassembly the disk is again washed, rinsed in acetone, and placed in a warm place to dry. The gross weight is generally obtained before the radioactivity is determined, although this is not essential.

Notes on the Analytic Procedure.—

Digestion: As often happens in the wet digestion of biologic tissues, there are occasional samples which precipitate sparingly soluble crystalline salts upon evaporation to small volume. When such occurred during this work, the solution and precipitate were transferred together to the centrifuge cone and the procedure was carried through as with the other samples. The presence of this material, which was always in very small amounts, apparently did not interfere with the results. To insure that no radioactive gold is lost, however, any insoluble material should be dissolved at some point between the time the carrier is added and the time reduction is completed, even though an extraneous precipitate appears later as it frequently does with urine and feces samples. This precipitate does not interfere with the electrodeposition. When octyl alcohol is used to prevent frothing, a waxy material is frequently formed which has a low melting point and which may not be observed until the solution is cooled to below room temperature. Precautions should be taken to obtain complete digestion of this organic matter.

Reduction: The use of both hydroxylamine and ferrous sulfate was investigated. The latter reduced the gold more rapidly than the former, and there was usually a small amount of unwetted gold floating on the meniscus after centrifuging. This gold was either lost upon decantation or had to be removed on a stirring rod and returned later. Reduction with hydroxylamine occurred in three distinct phases, namely, reduction to aurous, formation of colloidal gold, and finally, precipitation of the metal. Unwetted gold was not usually obtained when this reagent was employed, but the formation of a light gold mirror was observed frequently. When a mixture of both reductants was used, both phenomena were less pronounced, and the rate of reduction was about the same as with ferrous sulfate alone. The influence of acidity and salt concentration upon the rate of reduction and the character of precipitated gold was not investigated. A considerable range of conditions was encountered, but the results in all cases were satisfactory.

Solution of Gold in Aqua Regia: When mirror formation was encountered, the cone was placed in only about 1 inch of hot water to hasten the dissolving reaction. Sufficient aqua regia condensed on the wall of the cone to dissolve the mirror completely.

Addition of NaOH: If insoluble salts are present after digestion, a white precipitate is sometimes present or is formed at this point. If desired, this insoluble material may be removed by centrifuging before the solution is transferred to the electrodeposition cell. This was not done with the experiments reported.

The Cathode Disk: Both platinum and gold-plated copper disks may be cleaned by immersion in warm concentrated sulfuric acid-dichromate cleaning solution. With the latter type of disk, immersion should not be for longer than two to three minutes, since the plate does not offer sufficient protection to the copper to prevent its dissolving. Both types of disk must be thoroughly rinsed with water after this treatment. The platinum disks may be dried directly after washing but an acetone rinse is recommended first for the gold-plated copper. Heating the platinum disks over a burner in order to dry them is not recommended, since they then lose their temper and are more difficult to handle without bending. Early experiments carried out by Bertrand[10] indicated that plain copper disks lost rather than gained weight during electrodeposition. Without investigating this problem further, it was decided to try gold-plated copper disks in the hope that the formation of oxide film on the surface of the copper and attack of the copper by the cyanide plating solution could thus

be reduced or eliminated. The alternative was to use either gold or platinum disks, which, in view of the number needed and the difficulties involved in removing the radioactive layer, would not be economical. While the use of plated copper did not entirely eliminate the weighing error, the results obtained were satisfactory for this type of analysis. The thickness of the commercially made gold plate was not determined. The actual plating was done on strips of copper 14 by 1¾ by 0.002 inch. The 1½ inch disks were then punched out.

Electrodeposition: It was frequently observed that the plated disks in the assembled cells would discolor upon transference of the gold solutions to them. This occurrence was minimized, but not entirely eliminated, by adding the cyanide selution to either the cone or the electrodeposition cell before transference. The addition of the KCN directly to the cone prevents precipitation of auric hydroxide; the latter sometimes occurs upon allowing the solution to stand for several hours with NaOH alone. Neither the cause of the discoloration reaction nor its influence upon the gravimetric determination was investigated. The color of the gold plate on platinum disks was either yellow or light orange, while that on gold-plated copper varied from light rose to brown. The color in the latter case, as pointed out by Weisburg and Graham,* may be due to the presence of copper (from the cathode) in the plating solution. Gold plates on platinum frequently showed small blisters, in which case the deposit was easily broken loose by scratching. The use of a wetting agent such as one of the alkyl aryl sulfonates, as suggested by Hartshorn,[11] might eliminate this difficulty. Blistering was not observed with the plated copper disks, but the deposit on these disks could be rubbed off to an appreciable extent, perhaps because of the admixture of copper; hence highly active disks must be handled carefully in order to prevent contamination of counters, and so on, due to powdering off of small, unweighable, amounts of gold. The decision to use alkaline cyanide plating solutions rather than acid chloride was based upon the assumption that in the case of the latter there probably would be some attack of the platinum anode due to the formation of free chlorine and, therefore, deposition of platinum with the gold. The possibility of attack on the platinum by the cyanide, however, is not ruled out.

ANALYTIC RESULTS

The data shown in Table I should be divided into three groups. Determinations 77 through 84 were carried out with what is considered to be the proper amount of carrier gold to insure complete recovery of the radioactivity and at the same time to give a sufficient amount of deposit for gravimetric purpose, namely 10 milligrams. Determinations 85 through 92 were carried out with less than 10 mg. of carrier gold. With both of these groups, the various solutions were added directly to the assembled electrodeposition cells as follows: 5 ml. of 1N sodium hydroxide, 4 drops of hydrochloric acid, 1 drop of nitric acid, 2 ml. of 5 per cent potassium cyanide, the carrier gold solution, and the radiogold solution. Distilled water was added in each case to make a total volume of approximately 30 milliliters. Determinations 77 through 92 were, actually, eight sets of duplicates.

The third group comprises Determinations 93 through 96. This group of four identical determinations was carried out to test the recovery and electroplating procedures under carefully controlled conditions. For this purpose, three normal adult mice, weighing 20 to 25 grams each, were dry ashed in the muffle furnace at 600° C. The ash was taken up in aqua regia and the excess nitric acid removed, as outlined under Procedure. The resulting solution was diluted to 150 ml., and 10 ml. portions were used for each of these four determinations. The carrier and radioactive gold solutions were added, and the foregoing procedure was then followed.

* Page 204.[7]

TABLE I. RECOVERY OF RADIOACTIVE GOLD FROM SOLUTIONS CONTAINING VARYING AMOUNTS OF RADIOACTIVITY AND CARRIER

DETERMINATION	NORMAL GOLD ADDED (MG.)	RADIO GOLD ADDED (ML.)	INCREASE IN DISK WEIGHT (MG.)	RECOVERY OF NORMAL GOLD (%)	COUNTING DATA: COUNTS PER ML. Au^{198} (PER MIN.)	COUNTING DATA: COUNTER SHELF	COUNTING DATA: ELAPSED TIME (HR.)	EXTRAPOLATED COUNT SHELF 2 (TIME =0)	RECOVERY OF RADIOACTIVITY† (%)
77	10.3	1	10.3	100	340	4	2	2400	–
78	10.3	1	10.5	102	330	4	2	2350	–
79	10.3	2	10.5	102	1610	2	36	2400	–
80	10.3	2	10.4	101	1585	2	36	2350	–
81	10.3	5	10.2	99	330	4	2	2350	–
82	10.3	5	10.4	101	335	4	2	2400	–
83	10.3	10	10.4	101	330	4	3	2350	–
84	10.3	10	10.4	101	320	4	3	2300	–
85	0	2	–0.3	–	1480	2	35	2150*	91
86	0	2	–0.4	–	1480	2	35	2150*	91
87	1.0	2	1.0	100*	1400	2	45	2300*	98
88	1.0	2	0.9	90*	1400	2	45	2300*	98
89	2.1	2	1.9	90*	1385	2	45	2250*	96
90	2.1	2	2.1	100*	1435	2	45	2350*	100
91	5.1+	2	5.1	99	1450	2	45	2350	–
92	5.1+	2	5.2	101	1435	2	45	2350	–
93	10.3	2	10.6	103	1450	2	46	2350	100
94	10.3	2	10.5	102	1435	2	46	2350	100
95	10.3	2	10.6	103	1435	2	46	2350	100
96	10.3	2	10.5	102	1400	2	46	2300	98
Average				101				2350	

Count on shelf 4×6.9 (approx.) = count on shelf 2.
*Not included in the averages.
†Based upon 2,350 counts per milliliter per minute.
Half life of Au^{198} 2.7 days.

TABLE II. RECOVERY OF GOLD FROM ASHED BIOLOGIC TISSUE SOLUTIONS; 9.8 MG. OF CARRIER GOLD ADDED

ARTHRITIC PATIENT: TISSUE SAMPLE	SAMPLE WT. OR VOL.	Au^{197} MG.	RECOVERY %	RABBIT: TISSUE SAMPLE	SAMPLE WT. (GM.)	Au^{197} MG.	RECOVERY %
Synovia	0.33 Gm.	9.2	94	Kidney	5.0	9.6	98
Muscle	0.71 Gm.	9.8	100	Spleen	2.0	9.7	99
Superficial fascia and fat	1.22 Gm.	9.7	99	Testicle	2.8	9.7	99
				Articular cortex	2.6	9.6	98
Skin	0.31 Gm.	9.4	96	Articular cortex	3.0	9.8	100
Deep fascia	0.31 Gm.	9.8	100	Adrenal	0.2	9.7	99
Blood	2 Ml.	9.8	100	Liver	6.2	9.5	97
Blood	2 Ml.	9.7	99	Marrow	1.2	7.9	81*
Blood	2 Ml.	8.0	82	Lung	3.3	10.0	102
Blood	2 Ml.	9.4	96	Aorta	0.3	9.3	95
Blood	2 Ml.	9.5	97	Synovia	0.2	9.6	98
Blood	2 Ml.	9.1	93	Synovia	0.4	9.6	98
Blood	2 Ml.	9.3	95	Tendon	0.2	9.5	97
Blood	2 Ml.	9.3	95	Tendon	0.2	9.4	96
Blood	2 Ml.	9.1	93	Small intestine	2.8	9.4	96
Synovial fluid	1.5 Ml.	8.8	90	Bone	1.0	9.6	98
				Heart	3.1	9.8	100
Second day urine	100 Ml.	9.0	92	Muscle	4.1	6.5	67*
				Skin	0.8	9.8	100
First day feces	Total†	8.5	87				
Second day feces	Total†	9.4	96				
Average			94				98

*Electrodeposition cells leaked; values not included in the average.
†Weight not obtained.

Two typical sets of biologic data are given in Table II. These data were collected during the course of the work with arthritis carried out by Bertrand and co-workers.[12]

DISCUSSION

No explanation is offered for the 101 per cent average recovery of added gold, Table I. Since the error involved in biologic work is usually greater than the error which would be introduced by using the per cent carrier gold recovery as a factor in calculating the radiogold recovery, no further work was carried out to eliminate this discrepancy. The per cent recovery values shown in Table II therefore were used in calculating the original tissue gold activity[12] not reported herein.

One surprising result obtained, as shown in Table I (Determinations 85 and 86), was the 91 per cent recovery of radioactive gold when no carrier gold was added to the solution in the electrodeposition cell. The solution of radioactive gold contained 5 μg of metallic gold per milliliter in the form of sodium gold thiosulfate. The quantitative recovery of gold during electrodeposition, therefore, was greater than one would be led to expect, the loses in disk weight notwithstanding.

In carrying out the gold purification procedure with tissue digests from the arthritic patient, ferrous sulfate was used as a reductant. Trouble was experienced only with the urine and feces samples. Upon adding the reductant to the digested solutions from these samples, a red-brown flocculent precipitate was obtained in three of four cases. The fourth case appeared to give a normal precipitate, but unfortunately the sample was lost upon centrifuging, and a better observation could not be made. The abnormal precipitates were completely soluble in concentrated HCl, showing that reduction had not occurred. The addition of hydroxylamine to these samples gave normal precipitation of the gold. The use of the mixed reducing solution with these two types of sample is not recommended; hydroxylamine alone should be used.

The results with the rabbit tissue samples, also shown in Table II, were obtained using hydroxylamine alone as a reductant. All determinations given in Table II were carried out using routine or roughly quantitative transfer techniques.

SUMMARY

A method is described for the recovery and estimation of radioactive gold from biologic tissues.

The results obtained with this procedure are well within the allowable error for this type of determination.

Under a rather wide range of conditions, satisfactory results were obtained in all cases.

The method is rapid, requiring no special techniques, and the electrodeposition equipment is fairly simple.

The author is indebted to Dr. J. H. Lawrence, Dr. C. A. Tobias, and Dr. J. J. Bertrand for assistance and advice, and to Jean Luce for the digestion of the many biologic samples needed for this work.

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THE USE OF PRESERVED ERYTHROCYTES FOR THE DETECTION AND IDENTIFICATION OF Rh ANTIBODIES

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AN IMPORTANT requisite for any Rh testing laboratory is a constant supply of group O human erythrocytes of various Rh types. For the qualitative detection of Rh antibodies, cells of types CDE (Rh_1Rh_2) and cde (Rh negative) are highly desirable, while for accurate identification of the antibodies which are encountered, cells of Cde (Rh′), cDe (Rh_0), and cdE (Rh″), and occasionally other rare types must be available. Even the largest blood typing centers frequently have difficulty obtaining donors of the desired types, however, and to the smaller laboratory this difficulty may constitute an insurmountable handicap to adequate antibody testing.

We have attempted to solve this problem by preserving blood specimens in anticoagulant solutions. We have found, as has been reported by others, that when blood is mixed with an appropriate volume of sterile Alsever's or a.c.d. solution and is stored in the refrigerator, it keeps with a minimum of hemolysis for many weeks. In evaluating such preserved blood for our purposes there were two questions which demanded an answer. (1) Do the cells retain their agglutinability? (2) Do they retain their specificity? In a preliminary attempt to answer these questions, the following study was made.

A donor group A MN Rh_2rh (cDE/cde) was bled aseptically and 5 c.c. of the blood were mixed in each of a series of capped bottles with 5 c.c. of sterile Alsever's solution. One bottle was withheld for immediate study and the remainder were stored at refrigerator temperature (4° C.). At weekly intervals one bottle was removed from the refrigerator for study and discarded.

Each specimen was titrated against a standard anti-D (Rh_0) blocking serum of proved keeping qualities in the following manner:[1]

(1) An approximately 2 to 3 per cent suspension of cells was made in 30 per cent bovine albumin‡ by adding 2 or 3 drops of the blood-anticoagulant mixture to 1 c.c. of albumin.

(2) The standard anti-D (Rh_0) serum was serially diluted in normal AB serum and 1 drop of each dilution was placed in a series of small agglutination tubes.

(3) To each tube was added 1 drop of the cell suspension.

(4) All tubes were incubated in a 37° C. water bath, centrifuged three minutes at moderate speed, and examined under the dissecting microscope for agglutination which was recorded as 1 plus to four plus.

The titers obtained are recorded in Table I. It may be seen that there was only very little fluctuation in titer, probably due entirely to variations in technique, until the seventh week. At that time there began a definite decrease in titer which was proved by adequate controls not to be due to deterioration of the serum. It was at this time that the stored blood first showed gross hemolysis.

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‡Prepared specifically for Rh testing by the Armour Laboratories, Chicago, Ill.

TABLE I. TITER OF STANDARD ANTI-Rh_0 (D) SERUM AGAINST Rh_2rh (cDE/cde) CELLS STORED IN ALSEVER'S SOLUTION AT 4° C.

AGE OF BLOOD (WK.)	TUBES OF SERIAL DILUTION							TITER
	1	2	3	4	5	6	7	
0	4	4	4	3	2	1	–	32
1	4	3	3	2	1	1	–	32
2	4	4	3	2	1	–	–	16
3	4	4	3	3	2	1	–	32
4	4	4	3	2	1	–	–	16
5	4	4	3	2	1	–	–	16
6	4	4	3	2	1	–	–	16
7*	4	4	3	2	–	–	–	8
8*	4	2	–	–	–	–	–	2
9*	3	2	1	–	–	–	–	4
Control†	4	4	3	2	1	–	–	16

*Grossly hemolyzed.
†Fresh cells of same donor tested against stored serum at same time as 9-week-old cells.

A saline cell suspension of each specimen was likewise tested against the high-titered human anti-A and anti-B sera which are routinely used in this laboratory for Landsteiner grouping as well as against the routine Rh subtyping sera. In every case the reactions were exactly as they were when the blood was fresh. No false agglutination or false negative reactions occurred.

Similar observations have been made since in blood specimens of all of the Landsteiner types and a wide variety of Rh and Hr types. In the two years that Alsever's and a.c.d. solutions have been used in our laboratories, there has never been any evidence that cells preserved in this way are not perfectly reliable for Rh testing as long as there is no gross hemolysis. This period has varied in our hands between two and eight weeks, depending upon the care with which contamination is avoided and the amount of agitation to which the cells are subjected.

DISCUSSION

The advantage of a satisfactory preserving medium for blood cells for Rh testing is perfectly obvious. Since the introduction of the Alsever technique into our laboratories we have had little difficulty in keeping ourselves stocked with cells of all of the Rh subtypes which we have needed in our work. It is simple to keep a roster of patients and donors of various types, and at periods of a few weeks or a month to call in the appropriate individuals for renewal of the store of preserved bloods. We have found it most satisfactory to divide each donation between two or more bottles of anticoagulant, so that if one becomes contaminated or hemolyzed in handling, the supply of cells will not be lost entirely. When cells of some particular type are needed for testing, a few drops are drawn aseptically from the appropriate bottle and a cell suspension is made up in saline or albumin. If one is using a technique such as the slide test of Diamond and Abelson or the conglutination test of Wiener in which the presence of an aqueous solution of sodium citrate might interfere with the test, it is necessary to centrifuge the mixture, pour off the supernatant, and resuspend the cells in undiluted serum or plasma.

We feel that this method of preserving blood has become an indispensable adjunct to the proper functioning of our blood grouping laboratory.

SUMMARY

Alsever's and a.c.d. solutions have been found valuable for preserving whole blood of various antigenic types. Cell suspensions made from such preserved blood are satisfactory for testing sera for the corresponding Rh and Hr antibodies.

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METHODS TO INCREASE ACCURACY IN THE USE OF HAYEM'S SOLUTION FOR RED BLOOD COUNTS

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IN 1934 Bryan and Garrey[1] published a description of a rotor for the mechanical mixing of blood in diluting pipettes which affords more exact white counts than other methods of mixing. The erythrocytes, however, often were found to ball when diluted with the usual diluent for red blood counts, Hayem's solution. This effect was found to be due to the bichloride in the diluent, and Toison's fluid was therefore recommended for red counts.

Ch'u and Forkner[2] observed clumping of erythrocytes with the use of Hayem's solution in certain pathologic conditions even when the pipettes were shaken by hand, and they suggested substitution of Gower's solution to avoid the presence of bichloride.

Neither of these suggestions, as is also true of other substitutions that have been offered over the years, has received popular adoption. The reasons for this are several: either the diluents suggested were impractical for the general run of cases and laboratories, or the precautions offered in the use of them, as well as of Hayem's solution, proved inadequate upon wider usage, or custom on the part of the medical profession at large and the relative degree of satisfaction that has been afforded by the use of Hayem's solution determined that that should remain the diluent of choice for red counts.

Hayem[3] devised the formula which is now in common use as a diluent for red counts at a time when blood counts were beginning. His methods of obtaining and mixing blood, and even of superimposing it on the chamber for counting, were less refined than those employed today, and the demands for accuracy were correspondingly different. Nonetheless, use of the fluid has sufficiently stood the test of time to be continued regardless of the dissenting voices from period to period. As Ch'u and Forkner[2] have enumerated, the ideal diluent should preserve the cells and sharpen their visibility without danger of destroying any of them or of too greatly distorting them, should keep without deterioration, should eliminate danger of growth of organisms, and should permit satisfactory distribution of cells in the pipette and on the counting chamber. Hayem's solution fulfills all the criteria outlined for an ideal diluent except the indispensable one of dependably satisfactory distribution of cells. With mixing by hand, or with vigorous mechanical shaking, errors due to unsatisfactory distribution can usually be reduced, though not eliminated. With the advent of the mechanical rotor which was proved so satisfactory for white blood counts, which can rotate as many as eight pipettes simultaneously and is therefore desirable as a time-saving device for clinical and research laboratories.

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and which is now manufactured commercially, the inequalities of distribution of erythrocytes in Hayem's solution have become more evident and more in need of remedy.

In view of the fact that the suggestions already made for substitution of other diluents for red counts have failed to be adopted, it seemed preferable to attempt modification of Hayem's solution in such a manner as to eliminate the dangers to distribution inherent in it, rather than to encourage further use of different diluents. Such modification has been attained in one of two ways: either by reduction of the proportion of bichloride to as low as that recommended by Jörgensen,[4] together with careful control of the pH of the diluent between 5.0 and 7.0, or by addition of such colloids as gelatin or lecithin to Hayem's or Jörgensen's solution without concern for pH below 7.0. Ch'u and Forkner[2] found Jörgensen's solution unsatisfactory in the cases that they studied, but as the importance of the hydrogen-ion concentration in the use of Jörgensen's solution was not recognized at that time, it is probable that failure to control that factor featured in their findings.

The gentleness of the movements of the rotor of Bryan and Garrey served as a tool to show that the danger of clumping with Hayem's solution often is masked by other methods of mixing, but is actually inherent in the use of bichloride in diluents irrespective of the manner of mixing. The rotor permitted analysis of the causes that underlie the clumping and that feature in the remedies which assure against it. These studies are given in detail in a companion paper.[5] This report will be confined to the practical points in the preparation and use of the modifications of Hayem's solution that have been found to prevent clumping, and to comparison between red blood counts made with Hayem's solution and with the modified diluents.

METHODS

The formula for Jörgensen's[4] solution is as follows:

Na_2SO_4	2.5 Gm.
NaCl	0.5 Gm.
$HgCl_2$	0.05 Gm.
H_2O (distilled)	100 c.c.

The formula for Hayem's solution modified by the addition of gelatin is as follows:

Na_2SO_4	2.5 Gm.
NaCl	0.5 Gm.
$HgCl_2$	0.25 Gm.
Gelatin	0.01 Gm.
H_2O (distilled)	100 c.c.

The gelatin used was a powdered preparation.* Other preparations were not tried, but it is judged that any preparation of sufficient purity to rule out the presence of harmful electrolytes would be satisfactory. It should, of course, be kept dry for weighing.

Preparation of Hayem's solution containing gelatin is made most easily by dissolving: (1) the desired amounts of sodium sulfate, sodium chloride, and gelatin in one-half the de-

*Sold as Bacto Gelatin by Difco Laboratories, Detroit, Mich.

sired amount of water, warming if necessary to hasten solution of the gelatin, and (2) the desired amount of bichloride in the other half of the desired amount of water. When the two solutions are complete they should be mixed, with precautions to avoid foaming, and stored. Where large amounts of the diluent are desirable because of frequency of demand, it is helpful to make a sufficient supply of the double-strength bichloride solution and to mix this with fresh batches of the double-strength saline-gelatin mixture as desired. The double-strength bichloride solution keeps indefinitely in Pyrex glass and may be filtered, if necessary, before mixture with the solution containing gelatin. The mixed solution must not be filtered, but merely decanted, should the precipitate occur that is common in saline solutions of bichloride.

Both of the modified diluents should be kept in Pyrex containers since solution of the glass inevitably carries the danger of elevation of the pH to the point where clumping of the red cells cannot be prevented. Furthermore, even readjustment of the pH—if it has become elevated by solution from a container—is not sufficient to avoid clumping. This indicates, of course, that electrolytes dissolved from glass must have effects upon clumping in addition to those produced by increase in the pH.* It has been determined that either of the modified diluents can remain in contact with the particular types of diluting pipettes that were used for these experiments for as long as twenty-four hours without change in pH.

Red counts made with the modified diluents were compared with counts made with the standard Hayem's solution. The counts were carried out under experimental conditions that were planned to simulate conditions that might occur in routine clinical use. Judgment as to satisfactory distribution for blood counts was made, first and foremost, by microscopic inspection of the filled counting chambers. The counts were carried out merely for experimental assurance that they would, in reality, meet the usual standards for red blood counts. They were not planned primarily for a comparison on a statistical basis, and although the number of counts with any given diluent is considerable, the number for any given condition with that diluent is admittedly too few for satisfactory statistical analysis. When compared on that basis, nevertheless, the groups of counts with each of the diluents do show trends that are in agreement with the conclusions based on the microscopic studies. Therefore the significant data from the counts are presented in Table I and Fig. 1, and are discussed as probable statistical support of conclusions that depend primarily upon microscopic studies. As additional security a larger series of counts made with Hayem's solution alone and with Hayem's solution containing gelatin was obtained for a single experimental condition. This material is presented in the discussion of the experimental data.

The blood for all of the pipettes for any given series of counts was drawn at the same time from a freely flowing finger stick. The same subject and observer were used throughout. Both Haak and Trenner automatic pipettes were used in the comparison. No differences due to the model of pipette were found. The pipettes always were given ten quick shakes by hand immediately after filling. The mixing of the blood with diluent was carried out by different methods which were, in turn, employed for different periods of time. When the blood was mixed by rotation, a commercial model of the Bryan-Garrey rotor was used. It was tested against one of the original experimental models and was found to give identical results. When the blood was mixed by a mechanical shaker, an experimental model devised by Beard[6]† was employed. In some instances mixing was started at once. In others, the pipettes were allowed to stand for various intervals before mixing was started. These data for the different sets of counts are given in Table I. Levy-Hausser counting chambers with the improved Neubauer ruling were used throughout. Twenty or more drops always were expelled from the pipettes before the chambers were filled. When successive drops were to be counted from a single pipette, ten or more drops also were

*I wish to thank Dr. Ann Minot, Department of Biochemistry, Vanderbilt Medical School, Nashville, Tenn., for these determinations and for the helpful interest which initiated investigation of the causes which underlie the clumping of blood with Hayem's solution.[5]

†The model was made while Dr. Beard was connected with Vanderbilt Medical School, Nashville, Tenn. Similar models have been used in numerous laboratories through the courtesy of that school.

expelled between each filling and all chambers were filled in sequence. Those not to be counted at once were allowed to stand on moist paper under a bell jar.

There was a striking tendency for the red cells to become aligned with the lines of the chamber and to leave the centers of the small squares relatively depleted of cells when the diluent contained gelatin and the chamber was allowed to stand for any considerable time after filling. Since analysis of the data to follow indicates a gain in accuracy in

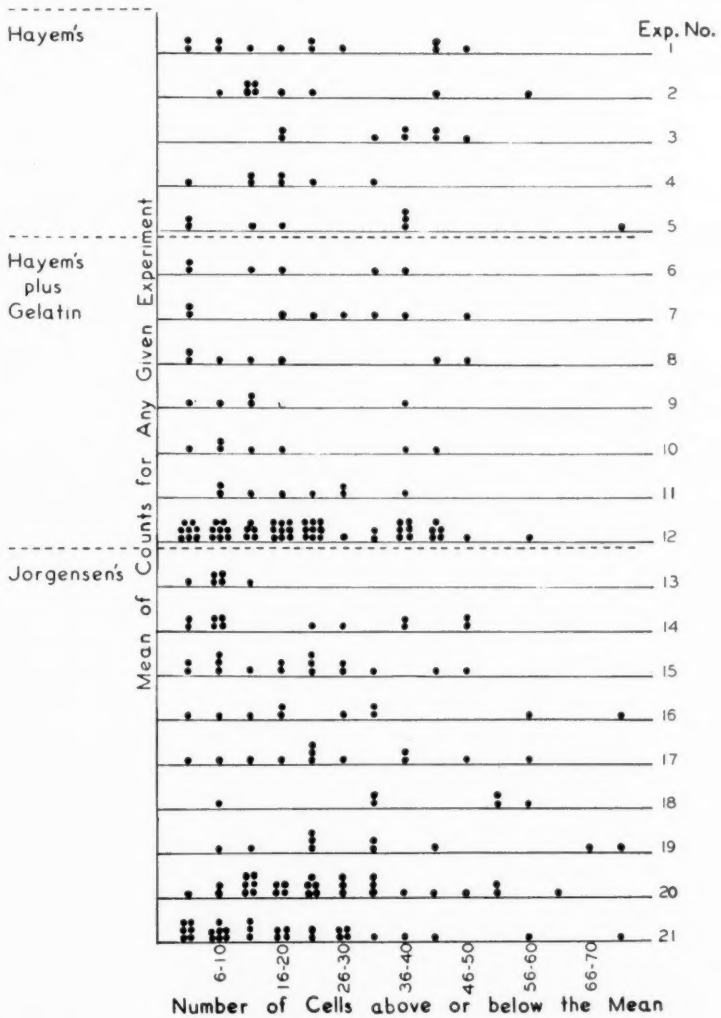

Fig. 1.—Scatter chart showing the number of counts in a series that fall within any given deviation of the mean of the series. Experimental conditions can be obtained by reference to Table I.

counts made in the presence of gelatin, this alignment of the red cells would seem to be unimportant to the accuracy of the counts and to represent simply a mutual shift in position of cells that had been well distributed, rather than a basic fault in distribution.

In conformity with the usual clinical routine, five small squares of the counting chamber, that is, one-fifth of a square millimeter, were included for each count. The values are

TABLE I. DATA FROM COUNTS OF RED BLOOD CELLS WITH DIFFERENT DILUENTS

EXPERIMENT	DILUENT	CONDITIONS OF EXPERIMENT	NUMBER OF PIPETTES	NUMBER OF COUNTS FROM EACH PIPETTE	TOTAL NUMBER OF COUNTS	MEAN OF ALL COUNTS	STANDARD DEVIATION OF INDIVIDUAL COUNTS FROM THE MEAN	STANDARD ERROR OF STANDARD DEVIATION
1	Hayem's	Pipettes stood 0-24 hr.; shaken by hand	6	2	12	413	26.5	5.4
2		Pipettes rotated 30 min.	9	1	9	433	27.5	6.5
3		Pipettes rotated 30 min.	8	1	8	447	37.2	9.3
4		Pipettes rotated 30 min.	7	1	7	445	19.0	5.1
5		Pipettes rotated 45-80 min.	8	1	8	444	35.3	8.8
6	Hayem's plus gelatin	Pipettes rotated 30 min.	6	1	6	429	22.4	6.5
7		Pipettes rotated 30 min.	8	1	8	460	27.7	6.9
8		Pipettes rotated 30 min.	7	1	7	449	25.3	6.8
9		Pipettes rotated 40 min.	6	1	6	438	20.0	5.8
10		Pipettes stood; then rotated 20-60 min.	8	1	8	478	23.2	5.8
11		Pipettes stood 1-3 hr.; then rotated 20-85 min.	8	1	8	450	22.9	5.7
12		Pipettes rotated 50 min.-22 hr.	7	8	55	431	25.4	2.4
13	Jörgensen's	Pipettes rotated 30 min.	6	1	6	441	8.2	2.4
14		Pipettes stood; then rotated 30 min.	6	2	12	423	27.7	5.7
15		Pipettes rotated 30 min.	8	2	16	461	24.0	4.2
16		Pipettes stood 3-5 hr.; then rotated 30 min.	5	2	10	472	35.6	8.0
17		Pipettes stood 30 min.-16 hr.; then rotated 30 min.	6	2	12	459	31.4	6.4
18		Pipettes rotated 30 min.-3 hr.	3	2	6	415	43.2	12.5
19		Pipettes rotated 1-6 hr.	5	2	10	461	39.9	8.9
20		Pipettes rotated 30 min.-5 hr.	4	6-8	30	464	29.6	3.8
21		Pipettes shaken mechanically 30 min.-5 hr.	4	8	32	465	24.9	3.1

tabulated as obtained, without use of the factor (10,000) necessary to convert them to the number of cells per cubic millimeter of blood. The mean total count for any given series of counts represents the average of all of the counts in the series. The deviations of each individual count in the series from this mean were used for the determinations of the standard deviations from the mean which are recorded in Table I and for the scatter plots illustrated in Fig. 1.

In making the counts with Hayem's solution a certain amount of selection was necessary. This was done by elimination of any preparation in which clumping was obvious upon inspection of the filled chamber under the microscope. The counts with Hayem's solution consequently represent the selectedly best preparations. In evaluating the data, therefore, it must be remembered that the agreement between individual counts made with Hayem's solution is better than would have been the case had all preparations been utilized. The same statements may be made of preparations made with Jörgensen's solution when the pipettes were allowed to stand, or to rotate, for a long while. When mixed immediately after filling, none needed to be discarded. None made with Hayem's solution containing gelatin needed to be discarded.

DISCUSSION OF EXPERIMENTAL DATA

Examination of the last two columns in the table, in which are tabulated the standard deviation of the individual counts of a given series from the mean of the series and the standard error of the standard deviation, supports a number of deductions. First, there is more variation in these values in the counts made with Hayem's solution alone than in those made with Hayem's solution to which gelatin was added; further, these values not only vary more in the counts made with Hayem's solution alone but also they are at times considerably greater than in the counts made with Hayem's solution containing gelatin. When one remembers that the counts made with Hayem's solution alone were made on selectedly good preparations these differences become still more significant. Second, while at first glance the values from the counts made with Jörgensen's solution vary as greatly as do those from the counts made with Hayem's solution, the values fall into two categories when the counts are grouped according to the manner in which the pipettes were handled after filling. If the pipettes were rotated immediately after filling and for only a relatively short period of time, the results were as satisfactory as those obtained by use of the Hayem's solution containing gelatin. If, on the other hand, the pipettes were allowed to stand, or were rotated for a long period of time, the values varied to the same degree as in the counts made with Hayem's solution alone. Furthermore, as was the case with counts made with Hayem's solution that did or did not contain gelatin, these variations are the more significant when one remembers that only the selectedly good preparations were counted when the pipettes stood or rotated for long periods, while all were counted otherwise. When the preparations were subjected to the more vigorous agitation afforded by the mechanical shaker, clumping was minimized and the counts were as accurate as those made with Hayem's solution containing gelatin, even when the pipettes were allowed to stand or shake for long periods. It should be emphasized here, nevertheless, that since the mechanical shaker has been shown to give less satisfactory distribution when used for white blood counts than does the rotor,[1] it is less desirable for complete blood counts than the rotor used in conjunction with a modification of the diluent for red counts which will obviate clumping.

Inspection of the scatter plots in Fig. 1, where the number of individual counts that deviate by graded amounts above or below their means are charted directly, corroborates the conclusions drawn from the table. It can be seen that more of the values fall near the line of the means and that the scatter is less extensive in the counts made with Hayem's solution containing gelatin, or with Jörgensen's solution with immediate and brief rotation of the pipettes, than in the counts made with Hayem's solution alone or with Jörgensen's solution under other conditions.

As mentioned previously, a larger series of counts carried out under a single experimental condition is offered as additional support to these deductions. The method of agitation that is most advantageous to the use of Hayem's solution was chosen for these counts. Difference between the values obtained

from the counts with Hayem's solution and those with the solution containing gelatin is consequently minimal, but even under this circumstance the values are in favor of the counts made with the protected solution, and the underlying potentialities for more erratic distribution with Hayem's fluid are still in evidence.

The pipettes were shaken vigorously by hand for three minutes before the chambers were filled instead of by mechanical rotation in order to avoid the necessity of selection of preparation for the counts to be made with Hayem's solution. All fillings of the chambers were then used indiscriminately and irrespective of the diluting fluid employed. The pipettes were allowed to stand from ten minutes to six hours before they were shaken. With these exceptions the technique was as previously described.

One hundred and four counts were made with each solution; eight were made from each pipette. The differences of the individual counts for each pipette from the mean were based on the mean of that pipette, rather than on the mean of all of the counts, in order to avoid the variations due to the pipette or to the filling which would be irrelevant to the present comparison. The differences thus obtained were used collectively for the remaining computations. Under these circumstances, the standard deviation of the counts made with Hayem's solution alone (pH 5.0) was 22.9 with a standard error of 1.6; the standard deviation of the counts made with Hayem's solution containing gelatin (pH 5.3) was 19.0 with a standard error of 1.3.

It is obvious from these data that Hayem's solution carries an inherent capacity for the clumping of red cells which is unpredictable and which requires selection of preparation and perhaps even of pathologic case.[2] This tendency is magnified when mixing is achieved by gentle rotation but is not caused by it, and affects the accuracy of counts even when it is not evident upon microscopic inspection. When gelatin is added to the diluent and the pH is below 7.0, the tendency is obviated, and counts, even unselected ones and those put under stresses which cause all preparations with Hayem's solution to clump, are correspondingly more dependable.

Accuracy also may be increased by reduction of the amount of bichloride to that in Jörgensen's solution, but only under restricted conditions. If the pH is kept within the confines of 5.0 to 7.0 and if the pipettes are rotated soon after filling and for not too long, this statement holds true. Otherwise the counts made with Jörgensen's solution are no more exact than those made with Hayem's solution. Vigorous shaking with less gentle movements than those of the rotor increases the accuracy, but the underlying liability to lesser degrees of clumping remains.

The use of Jörgensen's solution carries a second danger in addition to that of clumping which must be borne in mind in the case of pathologic blood specimens. In the studies that were carried out to explain the underlying factors that feature in the clumping of red cells in the presence of bichloride,[5] it was found that dilution of the proportion of bichloride to sufficiently low amounts facilitates lysis, especially if the pH is also low. Lysis of a few of the red

cells was observed in Jörgensen's solution with normal blood when the values for pH were below 5.0. With values above that, lysis was not detected in normal blood nor in a patient with congenital hemolytic icterus in relapse.* The saline fragility of this patient at the time of the observations was above 0.56, the red count, 2,560,000, the reticulocyte count, 12.8 per cent, and the icteric index, 15 units. The erythrocyte counts of this patient made from pipettes diluted with Jörgensen's solution and allowed to stand for as long as twenty-four hours before counting did not differ from those from similarly filled pipettes that were counted immediately, nor from pipettes that were diluted with Hayem's solution containing gelatin. Lysis must be detected by observation of fading cells or through comparison of counts made by methods in which there is no question of lysis, since the effect of bichloride upon any liberated hemoglobin conceals the reddening of the suspensoid which ordinarily announces hemolysis.

Berkson and co-workers[7, 8] do not state specifically what diluent they employed in their series of statistical studies concerning the limits of significance in red blood counts, but they do state that they used the methods in common practice. It is therefore assumed that they used Hayem's solution. Since the clumping that has been found to be an underlying tendency in the use of this diluent may often be so slight as to escape attention, it is reasonable to suppose that the values which these authors obtained are greater than would be the case had they used one of the modifications of Hayem's solution discussed in the present studies. This assumption is strengthened by the counts made in these studies. It will be seen from the table that when Hayem's solution alone was used, the standard deviations were at times as great as those found by Berkson and co-workers, namely 37.5 counted erythrocytes (375,000 total red blood cells). In the counts with Hayem's solution containing gelatin, or with Jörgensen's solution under controlled conditions, the values are decidedly lower.

CONCLUSIONS

Hayem's solution carries an inherent capacity for the balling or clumping of cells when used for red blood counts. The tendency toward clumping is magnified by gentle rotation but underlies all manners of mixing. It affects the dependability and accuracy of counts made with Hayem's solution.

The capacity for clumping inherent in Hayem's solution is due to the component of bichloride. It can be eliminated by decrease of the proportion of bichloride to that of Jörgensen's solution, together with control of the pH of the diluent between 5.0 and 7.0, or by addition of gelatin to Hayem's solution without concern for pH below 7.0.

Counts made with these modifications of Hayem's solution are subject to less deviation than are counts made with Hayem's solution alone under the same conditions.

*Case History No. 115,383, Vanderbilt Hospital, Nashville, Tenn. I am very grateful to Dr. Edgar Jones and Dr. Henry Warden, Department of Medicine, Vanderbilt Medical School, Nashville, Tenn., for the clinical data concerning this patient and for allowing me to make these studies. I am also very grateful to the Department of Social Service of the Vanderbilt Hospital for the interest which they exercised in helping me to contact this patient.

Use of Hayem's solution containing gelatin allows greater flexibility of experimental conditions without loss of protective power than does use of Jörgensen's solution. Addition of gelatin to Hayem's solution is, therefore, the modification preferred.

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A SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF URINARY BILIRUBIN

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A REVIEW of the literature on bile pigment metabolism and tests of hepatic function reveals that many quantitative tests for urinary bilirubin have been proposed. Probably the most satisfactory to date is that devised by Goodson and Sheard[1] in 1940 employing diazobenzenesulfonic acid. In 1941 Scott[2] employed the diazo reagent of Ehrlich and the unit system of van den Bergh in the quantitative determination of urinary bilirubin. His method is the most accurate, being sensitive to 0.02 mg. of bilirubin per 100 ml. of urine. It is, however, a procedure too difficult and time consuming to be employed as a routine clinical laboratory test. Gellis and Stokes[3] employed a modification of the methylene blue test of Franke[4] in their studies of infectious hepatitis in 1945. This was at best only a roughly quantitative test. Stokes and co-workers[5] in 1946 attempted to adapt the methylene blue test to the spectrophotometer but with little success, principally because the color produced in the methylene blue test is not the color of a true solution but a combination of colors. The barium-strip modification of Harrison's spot test was developed by Watson and Hawkinson[6] in 1946 as a semiquantitative test.

It becomes immediately evident that a simple, yet reasonably accurate, quantitative test of bilirubinuria is needed. The value of such a test in the diagnosis and prognosis of hepatic and prehepatic hyperbilirubinemia and in the diagnosis of hepatitis in the preicteric stage is obvious. In order to meet this need the following method is elaborated. It is based on the oxidation of bilirubin to a green derivative and the estimation of the latter spectrophotometrically at a wave length of 670 millimicrons.

METHOD

In this procedure a modified Fouchet's reagent is utilized as an oxidizing agent. This is prepared with 6.25 Gm. of trichloracetic acid and 2 ml. of a 10 per cent ferric chloride solution diluted to 200 ml. with distilled water. A Coleman Junior spectrophotometer is used with 15 mm. cuvettes and adapter. A standard bilirubin solution is prepared by dissolving 10 mg. of pure bilirubin in 100 ml. of chloroform. To 20 ml. of this standard solution are added 80 ml. of 95 per cent ethyl alcohol resulting in a 2 mg. per cent solution of bilirubin. Further dilutions of the latter solution are made as desired with 95 per cent ethyl alcohol. Into a 15 mm. cuvette are placed 4 ml. of the chloroform alcohol solution of bilirubin of known concentration, 4 ml. of 95 per cent ethyl alcohol, and 2 ml. of the modified Fouchet's reagent. A reference tube is prepared by adding to 4 ml. of 20 per cent chloroform alcohol solution 4 ml. of 95 per cent ethyl alcohol and 2 ml. of 3.125 per cent trichloracetic acid. Ten minutes are allowed to elapse after the addition of the modified Fouchet's reagent to permit the full development of the green color. The spectrophotometer is set at a wave length of 670 mμ and at 100 per cent transmission with the reference tube. A reading then is taken

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with the bilirubin solution and plotted on semilogarithmic graph paper using per cent transmission as the abscissa and concentration of bilirubin in milligrams per 100 ml. as the ordinate. The point is then connected by a straight line to the point—concentration equals 0, transmission equals 100 per cent. The Lambert-Beer Law, $c = -K \log T$, will be expressed by the curve represented by this straight line (Fig. 1). From this curve the concentration of any similarily oxidized bilirubin solution can be determined if the percentage of transmission is known.

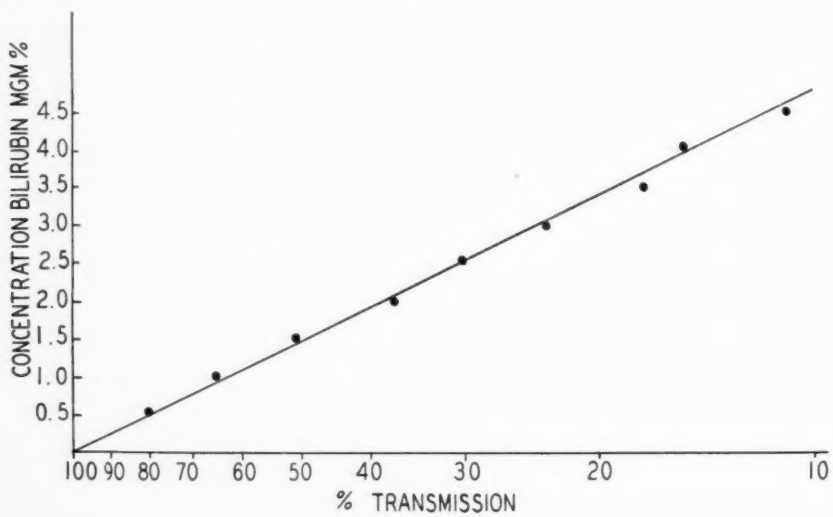

Fig. 1.

The wave length of 670 mμ was chosen after the preparation of a spectrotransmission curve using a 2 mg. per cent alcohol chloroform solution of bilirubin treated with the reagent as described. The reference was prepared with 8 ml. of 20 per cent chloroform alcohol solution plus 2 ml. of modified Fouchet's reagent. This curve was plotted from readings made at 25 mμ intervals from 400 through 700 millimicrons. From this curve it was evident that the ideal wave length was from 650 to 700 millimicrons. After readings were made at 5 mμ intervals from 650 to 700 mμ, it was found that a wave length of 670 mμ was most desirable.

PROCEDURE FOR DETERMINING URINARY BILIRUBIN

To 4 ml. of undiluted urine in a 15 mm. cuvette are added 4 ml. of 95 per cent ethyl alcohol and 2 ml. of the modified Fouchet's reagent. A reference tube is prepared with 4 ml. of urine plus 4 ml. of 95 per cent alcohol and 2 ml. of 3.125 per cent trichloracetic acid. After ten minutes a reading is taken in the spectrophotometer at a wave length of 670 mμ, and the concentration of bilirubin is determined from the previously prepared graph.

DISCUSSION

This procedure provides a simple quantitative method for the determination of urinary bilirubin. Attempts at recovery of bilirubin added to the urine were highly successful as can be seen in Table I. The bilirubin was added to the urine in the form of icteric serum, the concentration of which was determined spectrophotometrically by the method of Malloy and Evelyn.[7] The data provided by these determinations serve to confirm the validity of the curve prepared in the foregoing.

TABLE I. VARIATION AND PER CENT RECOVERY BY SPECTROPHOTOMETRIC METHOD OF BILIRUBIN ADDED TO URINE

CONCENTRATION OF BILIRUBIN (MG./ML.)	RECOVERY OF BILIRUBIN (MG./ML.)	VARIATION (MG./ML.)	RECOVERY (%)
1.30	1.25	-0.05	96
1.65	1.50	-0.15	91
0.93	0.90	-0.03	97
1.05	1.50	-0.10	90
0.83	0.80	-0.03	96
0.90	0.85	-0.05	94
1.20	1.15	-0.05	96
0.50	0.50	-0.00	100
0.60	0.60	-0.00	100
0.70	0.65	-0.10	93
0.85	0.75	-0.10	88
2.45	2.35	-0.19	96
2.85	2.65	-0.15	93
3.00	2.85	-0.10	95
3.50	3.40	-0.15	97
4.05	3.90	-0.05	96
5.00	4.95	-0.10	99
5.55	5.45	0.00	98
6.00	6.00	-0.15	100
6.50	6.35	-0.02	98
1.70	1.68	-0.02	99
0.16	0.14	-0.02	87
0.77	0.75	-0.02	97
0.39	0.37	-0.06	95
1.00	0.94	-0.09	94
1.50	1.41	-0.05	94
Mean		-0.07	95

The original Fouchet's reagent will yield a green color if used in this procedure. This, however, has a pH of 1 and it was found that a 25 per cent trichloracetic acid solution (pH 1) if used as a reference also would yield a green color. When the pH of the reagent and reference solution was brought up to 4, no oxidation of the bilirubin to a green derivative took place in the reference tube. The oxidation at this pH apparently is dependent on the ferric chloride.

By using an equal volume of urine in the reference tube any extraneous color resulting from other chromogens is compensated for. The alcohol in no way enhances or interferes with the production of the green color. It has been introduced to free any bilirubin globin that might be present in a proteinuria.

The normal urinary bilirubin concentration has not been studied adequately, but the preliminary studies on random samples of urine of normal patients done in this laboratory agree with those of Naumann[8] who found it to be less than 0.5 mg. per cent.

To date, salicylates are the only substances found to interfere with this test. If present in sufficient concentration they will produce a pink color when the reagent is added.

SUMMARY

A simple and accurate spectrophotometric method for the quantitative determination of urinary bilirubin has been presented. This test is dependent on the production of a green color resulting from the oxidation of bilirubin with an acid ferric chloride reagent. The choice of optimal wave length and the preparation of a standard bilirubin solution are discussed.

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A SCHOLANDER MICROMETER BURETTE OF SIMPLE CONSTRUCTION

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THE Scholander micrometer burette has been of value for the precise measurement of the delivery of small volumes of liquid. The original design[1, 2] specified a micrometer, the anvil of which had to be annealed,

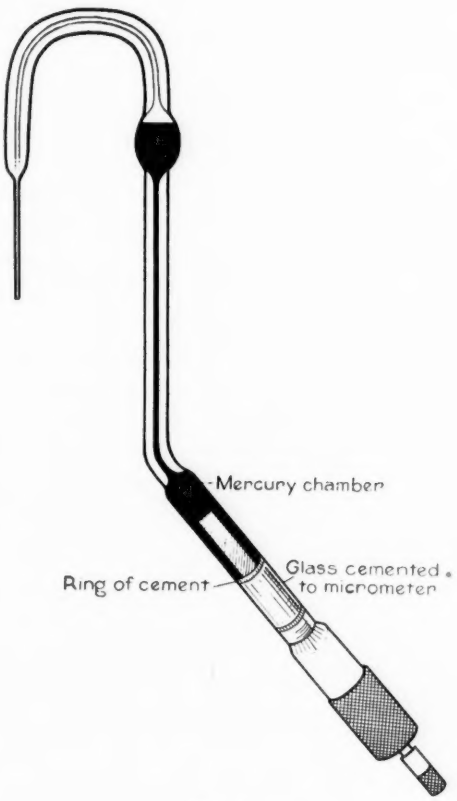

Fig. 1.

drilled, and threaded. The use of a micrometer head and a minor rearrangement in design eliminates all machine work and reduces glass blowing to a minimum. The result is a simple apparatus easily constructed by anyone with a knowledge of glass blowing at a fraction of the usual cost.

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The micrometer head used is a Brown & Sharpe No. 294 RS. The glass part is made of 1 mm. Pyrex capillary tubing and standard 11 mm. Pyrex tubing. The latter has a normal internal diameter of 9 mm. with a variation of 0.5 millimeter. In order to obtain a close fit between the 11 mm. tubing and the micrometer head, the internal diameter of the tubing should be smaller than the shoulder to be inserted. This diameter is then enlarged to the proper size by grinding it internally with emery paper wrapped around a wooden dowel rod until a close fit is obtained. Small variations in diameter can be taken care of by the cementing process.

In order to obtain a satisfactory cemented seal, both parts should be heated to about 145° C., preferably in a thermostatic oven. The inside of the lower end of the mercury chamber is coated with de Khotinsky cement, which will melt at the temperature given. The shoulder of the micrometer head then is fully inserted into the mercury chamber. If this is done properly, a continuous ring of cement will form at the end of the micrometer, effectively sealing off from the mercury chamber any air spaces which may have formed between the metal and the glass.

The seal must be carefully inspected after the apparatus has cooled. Should the ring of cement not be continuous, the defect can be corrected by introducing a small piece of the cement into the mercury chamber through the micrometer head at the proper place and then reheating the apparatus.

The other details of construction will be apparent from the drawing and need no further description.

We have found that heavy grease in the spindle, as suggested by Scholander, occasionally traps air bubbles which are forced into the mercury chamber by the movement of the spindle. We use a small amount of light machine oil to lubricate the micrometer threads. The hydrostatic head of mercury above the chamber effectively prevents air from entering along the spindle. It is essential to maintain this head at 5 cm. or more. The glass tip of the burette should be short enough to avoid a siphoning effect.

The burette described delivers volumes as accurately as the original model of Scholander.

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A MODIFIED TECHNIQUE FOR THE EXAMINATION OF BODY FLUIDS

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IN SOME instances a diagnosis of malignant tumor can be made by examining the cellular content of centrifuged sediment from body fluids, but the technical difficulty of obtaining a maximal amount of sediment has limited the usefulness of these studies. Schenken and McCord[1] suggested the use of collodion bags suspended in glass centrifuge tubes. Marcuse and Coulter[2] reported

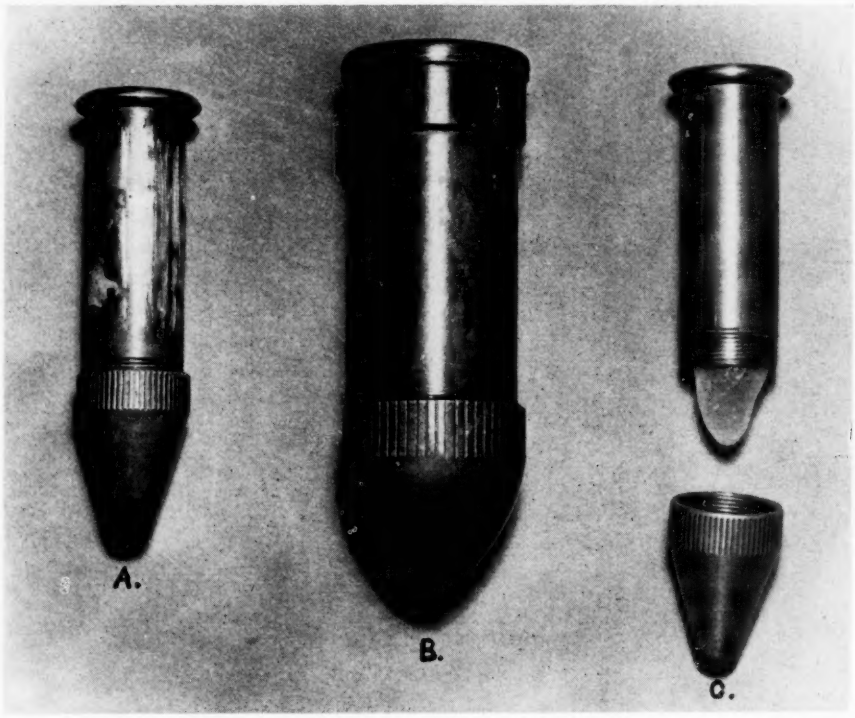

Fig. 1.—Metal centrifuge tubes used to aid in the study of body fluids. *A* and *B* show the intact tubes of two sizes. *C* illustrates the metal tip removed and the appearance of the collodion bag within the tube.

the use of a metal centrifuge tube made up of three separate parts which simplifies the removal of the sediment. The technique described requires twenty-four to thirty-six hours for completion of the examination. It was decided to combine the advantages of these two devices in an effort to make the study of body fluids more simple and rapid.

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The bottoms were removed from two sets of standard size metal centrifuge tubes and threaded to receive detachable tips. Each tube of the smaller set holds 15 c.c. and each of the larger set accommodates 50 c.c. (see Fig. 1).

In preparation for use, the tip is first detached and a thin film of stopcock grease applied to the inner surface of the tip and to the lower two inches of the inner surface of the barrel. The tip is screwed tightly to the barrel and 2 or 3 c.c. of moderately thick collodion is poured into the tube. With the tube held horizontally and slowly rotated, the entire inner surface is evenly coated with collodion. The excess is drained from the tube and the collodion is partially dried to form a stable film. It is advisable to determine by experience the necessary thickness of the collodion membrane.

The specimen is poured into the tubes and centrifuged at 1,500 revolutions per minute for fifteen to twenty minutes.* The detachable metal tip is then slowly unscrewed, leaving the intact collodion sac containing the sediment suspended from the barrel of the tube. A small incision just above the level of the sediment will allow the supernatant fluid to drain with a minimum of turbulence. The collodion tip is then cut off and dropped into Bouin's solution or formalin. After fixation for at least one hour, the block of sediment can be dehydrated, embedded in paraffin, and sectioned like tissue.

If desired, the sediment can be smeared on clean glass slides, fixed immediately in a 1:1 mixture of 95 per cent alcohol and ether, and stained by the Papanicolaou technique. In these cases the collodion tip is removed, inverted on a finger, and the inner surface rubbed on a slide. Even when no sediment is visible, a fair concentration of the cells often can be seen on the smear.

This technique has been especially fruitful in the study of urinary tract lesions, since renal tumors have been detected by examination of small volume washings of cells from the kidney pelvis. It also has been used to concentrate the cells in pleural and ascitic fluids, secretions aspirated from the bronchial tree, and gastric washings.

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*If the volume of body fluid is too great to be accommodated by the metal tubes it can be centrifuged in large glass containers and most of the supernatant fluid decanted. The liquid containing the sediment then can be transferred to the special tubes.